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CONTRIBUTIONS TO THE PHYSIOLOGY OF PHOSPHORUS AND CALCIUM METABOLISM AS RELATED TO MILK SECRETION.*

By EDWARD B. MEIGS, N. R. BLATHERWICK, and C. A. CARY.

(From the Research Laboratories of the Dairy Division, Bureau of Animal Industry, United States Department of Agriculture.)

(Received for publication, September 26, 1918.)

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INTRODUCTION.

For the development of our knowledge of the physiology of milk secretion, it is desirable that we should discover the precursors in the blood of the various constituents of milk. Very considerable progress has already been made in this field; it seems to us to be peculiarly accessible to experimental investigation, and we have devoted a good deal of time to working in it.

When the precursors of the milk constituents are known, it will be desirable to discover how their concentration in the blood is maintained against the great demand made for them by the mammary glands of heavily milking animals. We believe that light can be thrown on this subject by studying the concentration of certain materials in the blood under various conditions, and we have made a more or less extended study along these lines. We have paid particular attention to phosphorus and calcium, but we have studied also other constituents of the blood, when this promised further light on the problems in hand.

EXPERIMENTAL METHODS.

Our experiments have consisted in analyzing the blood and plasma obtained from the jugular or from the abdominal subcutaneous vein of intact normal or nearly normal cattle. The blood samples were obtained by means of a small trochar and cannula inserted through the skin into the vein. In expert hands the operation is an extremely simple one; it has never, in our experience, had any perceptible effect on the health or on the milk yield of the animals subjected to it. Our only antiseptic precaution has been to wash the skin and hair of the subject near the seat of operation, as well as the trochar and cannula, with 5 per cent carbolic acid solution.

The details of our experimental procedures have naturally varied from time to time. In describing them it will be most convenient to lay the chief emphasis on the routine which we consider the best at the present time, and later to add remarks on the changes that we have made in cases where this seems to be worth while.

There has not been, up to the present time, very much work done on the variations which occur in the concentration of the ash constituents of tissues, and particularly of the calcium and phosphorus of the blood, under normal conditions. In such work it is desirable to make a great many analyses and to be able to detect even small variations. In addition, a long experiment may often be rendered completely valueless or even positively misleading, if a single pair of incorrect results is obtained at some

critical point in its course. In beginning such work, therefore, it is important to develop methods of the highest possible accuracy, certainty, and rapidity.

We have tried a number of the methods recommended in the standard text-books and found them unsatisfactory; and the methods which we now use have been developed by a process of selection, and as the result of considerable experience. We think it advisable, therefore, to report them in some detail.

Collection of Blood; Prevention of Coagulation.

Our blood samples have been collected in clean glass bottles in which had previously been placed some reagent to prevent clotting. When we wish to analyze for phosphorus only, sodium oxalate is placed in the bottle in such quantity that each 100 cc. of blood collected will be mixed with 0.1 gm. of the salt; where calcium analyses are to be made, 1 cc. of saturated sodium citrate solution is mixed with each 100 cc. of blood. It is, of course, impossible to use plasma from oxalated blood for calcium analyses, as most of the plasma calcium is precipitated by the oxalate, and thrown down with the corpuscles during the centrifuging.

We have been able to show that oxalate does not interfere with phosphorus analyses as carried out by our methods and that citrate does not interfere with analyses for either calcium or phosphorus. We found it practicable to prevent clotting by drawing our blood into cooled vessels lined with paraffin and by keeping the blood and plasma in cooled paraffined vessels during the centrifuging and until we were ready to use it. In still other instances we prevented clotting by the use of hirudin. The blood and plasma kept fluid by the first of these methods may be called "natural" blood and plasma. It was found that if different portions of the same sample of blood were kept fluid by the "natural" method, by hirudin, and by citrate, the portions of plasma obtained from them all gave similar results for calcium and phosphorus; and that the portions of plasma obtained from "natural," hirudinized, citrated, and oxalated blood all gave similar results for phosphorus.

Centrifugation of Blood.

Our blood samples have been centrifuged, as a matter of routine, for 20 minutes at between 3,000 and 3,500 revolutions per minute, and in glass vessels marked so that the volume of the corpuscles could be read off when the centrifugation was finished. We found that longer centrifuging at higher speeds did not reduce the volume of the corpuscles any further.

Solutions and Reagents.

We have been in doubt as to what is meant in text-books of chemistry when one is told to make up a 10 per cent solution or a 25 per cent solution. Our procedures may, therefore, occasionally be somewhat differ-

ent in detail from what was intended in the descriptions from which we have taken them. But, as a preliminary step to adopting any method of analysis, we have naturally carried out a number of check analyses on known amounts of chemically pure snbstances; and, if our results were not satisfactory at first, we have varied our procedure until they became so. In describing our own solutions, we shall speak generally in terms of solutions by weight. By a "10 per cent hydrochloric acid solution," for example, we mean a solution of which 100 gm. contain 10 gm. of HCl and 90 gm. of water. In the case of salts which contain water of crystallization we shall give the strengths of our solutions in terms of the water-free salt.

Method of Ashing Material.

We have analyzed our samples of blood and plasma for calcium, total phosphorus, lipoid and inorganic phosphorus, and nitrogen, and in a few cases for total fat.

For the calcium and total phosphorus analyses the blood or plasma has been ashed with acid mixture, essentially as recommended by Abderhalden.[1] When this procedure is carried out with plasma, the concentrated sulfuric acid ash is always quite clear and colorless; in the case of blood, on the other hand, the concentrated ash contains a whitish precipitate, which dissolves in water and gives a positive test with potassium sulfocyanate, and which, we have no doubt, is ferric sulfate. It dissolves when the acid ash is boiled with water, leaving a very faintly yellow color; and it does not interfere with the calcium and phosphorus analyses.

Determination of Calcium.

Our method of determining the calcium in blood and plasma consists in a combination of parts of the procedure recommended by Abderhalden[2] with parts of McCrudden's method.[3] 30 to 50 cc. of blood or plasma are ashed with 25 cc. of acid mixture,[4] and the acid ash is finally boiled with about 25 cc. of water. The mixture is then transferred with the aid of a little water from the Kjeldahl flask used for ashing to a beaker, five times the volume of the mixture and washings in 95 per cent alcohol is added, and the whole is allowed to stand over night. The calcium sulfate so precipitated is filtered off, and washed three or four times with 95 per cent alcohol; the filtrate and washings may be used for the determination of total phosphorus (see below).

The filter paper with the calcium sulfate precipitate is dried and ashed in a platinum crucible and dissolved in approximately 35 per cent HCl by

[1] Abderhalden, E., Handb. biochem. Arbeitsmethoden, Berlin, 1910, i, 385–388.

[2] Abderhalden, Handb. biochem. Arbeitsmethoden, Berlin, 1910, i, 413.

[3] McCrudden, F. H., *J. Biol. Chem.*, 1911–12, x, 187.

[4] Equal parts by volume of 95 per cent H_2SO_4 and 70 per cent HNO_3.

digestion for 45 minutes at from 50–75°. The HCl solution so obtained is diluted with water, filtered into a beaker, and made up to about 75 cc. with water. One drop of 0.006 per cent phenolsulfonephthalein is added, and then 28 per cent NH_3 until the color changes through yellow to pink. The mixture is cooled and 10 per cent HCl is added drop by drop until the color changes back to clear yellow. 10 cc. of 0.5 N HCl and 10 cc. of 1.75 per cent $H_2C_2O_4$ are then added; the mixture is heated to boiling, then cooled to 50° or below; 10 cc. of 10 per cent $NaC_2H_3O_2$ are added, and the mixture is allowed to stand about 30 minutes at between 50 and 75°, and then over night at room temperature. It is filtered through a small filter paper, and the precipitate is washed free of chlorides with 0.4 per cent $(NH_4)_2C_2O_4$, and then washed free of soluble oxalate with water. Finally it is dissolved in about 20 cc. of warm approximately 2 N H_2SO_4, and washed through the filter with water to about 100 cc. It is heated to 70° and immediately titrated with 0.02 N $KMnO_4$, the calcium being calculated from the amount of standard permanganate solution required.

We have standardized our permanganate solutions frequently against known oxalate solutions. We found, however, that if the permanganate is carefully made up and kept in clean glass vessels, its strength does not change very much even in several months.

Abderhalden recommends weighing the calcium sulfate from the acid ash, either after one[4] or after two[5] precipitations with alcohol. We tried both these procedures and found them highly unsatisfactory for blood and rather unsatisfactory for plasma. We also tried various other methods of calcium analysis, such as drying our material and ashing it in platinum over a flame, and weighing the calcium as oxide or oxalate. These methods are, of course, capable of giving accurate results, but we found them decidedly more troublesome than the procedure described above, which is satisfactory both for blood and plasma.

Determination of Total Phosphorus.

When we wished to determine the calcium and phosphorus in the same sample of material, the alcoholic filtrate from the calcium sulfate precipitation (see above) was placed in a Kjeldahl flask and evaporated on the steam bath until the alcohol was nearly or completely driven off.[6] The concentrated filtrate was boiled until it became dark, and 5 cc. of 70 per cent HNO_3 were allowed to flow into it drop by drop while it was still boiling. The boiling was continued for about 20 minutes more, and the sulfuric acid ash so obtained was treated as described below.

When phosphorus alone was to be determined, the sample (20 to 35 cc. of blood or 30 to 50 cc. of plasma) was ashed with 25 cc. of acid mixture in

[5] Abderhalden, Handb. biochem. Arbeitsmethoden, Berlin, 1912, vi, 381.

[6] Evaporation from a flask may be very much hastened by inserting a glass tube down its neck nearly to the surface of the liquid to be evaporated, and sucking air through this by means of the vacuum pump.

the same manner as for the calcium determination. The sulfuric acid ash so obtained may be regarded as equivalent, so far as phosphorus is concerned, to the ash obtained from the alcoholic filtrate of the calcium determination, as described in the preceding paragraph, and from this point the description applies to both.

The concentrated sulfuric acid ash is boiled with about 30 cc. of water for a few minutes and then transferred to a beaker and diluted with water to 150 cc. 50 cc. of 33 per cent NH_4NO_3 are added, and the mixture is heated to boiling. At the same time 40 cc. of 8.6 per cent $(NH_4)_6Mo_7O_{24}$ are brought to boiling and poured into the phosphorus mixture. The resulting precipitation is again brought to a boil and stirred about 2 minutes; it is then allowed to stand 10 minutes or as much longer as may be convenient.[7]

The ammonium phosphomolybdate is filtered by decantation and washed once with 25 cc. of wash liquid;[8] then dissolved in 5 cc. of 10 per cent NH_3; 10 cc. of 25 per cent NH_4NO_3, 15 cc. of water, and 1 cc. of 2.7 per cent $(NH_4)_6Mo_7O_{24}$ are added, the mixture is brought to boiling, and reprecipitated by the addition of 10 cc. of boiling 31 per cent HNO_3; it is stirred for 2 minutes and then allowed to stand 10 minutes or more. It is again filtered by decantation, and the precipitate is dissolved without further washing in 12 cc. of 2.5 per cent NH_3, to which about 20 cc. of water are added. To the resulting solution 35 per cent HCl is added drop by drop until the precipitate resulting from one of the drops dissolves only slowly on stirring, and there are then added 2 cc. of magnesia mixture[9] and one drop of 1 per cent phenolphthalein. The solution is brought to a boil and 2 5 per cent NH_3 is added quickly from a pipette until a faint pink color appears; it usually requires 5 to 10 cc. The mixture is cooled to somewhat below room temperature, as a result of which numerous crystals of $MgNH_4PO_4$ should appear. About 10 cc. of 28 per cent NH_3 are added, and the mixture is allowed to stand 10 minutes more, or as much longer as may be convenient. It is filtered through a weighed Gooch crucible, the precipitate washed free of chlorides with 2.5 per cent NH_3, dried, and ignited to $Mg_2P_2O_7$; and the phosphorus is calculated from the weight of the pyrophosphate.

---

[7] The part of the procedure so far described is, in general, taken from Abderhalden (Handb. biochem. Arbeitsmethoden, Berlin, 1910, i, 420). Abderhalden, however, recommends heating the ammonium phosphomolybdate precipitation only to 70–80°, while we found that the phosphorus did not all come down unless it was heated to boiling as described above. Abderhalden recommends carrying the analysis further by the titration method, which we tried and found not entirely satisfactory. From this point in our analysis, we followed in general the procedure recommended by Treadwell (Analytical chemistry, New York 2nd edition, 1910, ii, 397–398).

[8] NH_4NO_3, 100 gm.; 70 per cent HNO_3, 80 cc.; water, 2 liters.

[9] $MgCl_26H_2O$, 110 gm.; NH_4Cl, 210 gm.; 35 per cent HCl, 4 cc.; water, 2 liters.

Determination of Inorganic Phosphorus.

The inorganic phosphate of plasma has usually been determined by a method developed by one of us[10] from a brief description given by Greenwald.[11]

To 80 cc. of plasma are added 160 cc. of water, 80 drops 0.1 N HCl, 4 cc. of saturated NH_4Cl, 1.6 cc. of 7.7 per cent $MgCl_2$, 32 cc. of 2.8 per cent NH_3, and the mixture is allowed to stand over night, preferably at about 5°C. If smaller quantities of plasma are taken, all the above mentioned reagents are reduced proportionally. The mixture is filtered, and the precipitate is washed three or four times with 2.5 per cent NH_3, twice with 95 per cent alcohol, and twice with ether. It is then dissolved from the filter paper into a beaker with 15 cc. of 8 per cent HNO_3, 10 cc. of 31 per cent HNO_3, and 45 cc. of water; 10 cc. of 25 per cent NH_4NO_3 and 30 cc. of 2.7 per cent $(NH_4)_6Mo_7O_{24}$ are added, the mixture is heated for an hour or so to from 50–75°, and then allowed to stand over night at room temperature. (The precipitate will come down completely without heating, if allowed to stand 48 hours at room temperature.) The mixture is filtered by decantation, and the analysis is carried to completion exactly as in the case of the determination of total phosphorus after the filtration of the second ammonium phosphomolybdate precipitation (see above).

In a few instances we determined the inorganic phosphate of plasma by the method described by Embden and his collaborators[12] for muscle juice. The same samples of plasma were analyzed by this method and by the one which has just been described and the results are given below.

Inorganic Phosphate in Plasma. Mg. per 100 Gm. of Plasma.

Plasma.	Determination.	Greenwald method.	Embden method.
		mg.	*mg.*
1	I	4.26	4.49
	II	4.30	4.49
	Average.....	4.28	4.49
2	I	5.89	6.07
	II	5.80	6.30
	Average.....	5.84	6.18
3	I	3.72	4.13
	II	3.99	3.96
	Average.....	3.85	4.04
4	I	4.66	4.85
	II	4.80	4.61
	Average.....	4.73	4.73

[10] N. R. Blatherwick.

[11] Greenwald, I., *J. Biol. Chem.*, 1916, xxv, 431.

[12] Embden, G., Griesbach, W., and Schmitz, E., *Z. physiol. Chem.*, 1914–15, xciii, 1.

In two other cases the plasma was analyzed immediately by the Greenwald method and after it had stood for some time, by the Embden method. The results were as follows:

	Greenwald method.		Embden method.
		mg.	*mg.*
Plasma 5. Analyzed by Embden method after standing 4 days.	I	6.34	7.00
	II	6.25	6.91
	Average.	6.29	6.95
Plasma 6. Analyzed by Embden method after standing 1 day.	I	4.13	4.40
	II	3.89	4.66
	Average.	4.01	4.53

For the first four samples of plasma the figures obtained by the Embden method average 0.18 mg. per 100 gm. of plasma higher than those obtained by the Greenwald method. This difference is very small; the question of its significance will be discussed later.

In the last two samples of plasma, which were analyzed immediately by the Greenwald method, and not until after standing some time by the Embden method, the differences in the figures obtained by the two methods are greater than in the other four. These results indicate that the phosphatide of the plasma tends to decompose somewhat on standing; and that, in cases where the highest accuracy is desired, the samples should be analyzed immediately.

Determination of Lipoid Phosphorus.

The great majority of our determinations of lipoid phosphorus have been carried out by the nephelometric micro method, which was suggested some time ago by Pouget and Chouchak,[13] and has since been developed by Greenwald,[14] Kober,[15] and Bloor.[16] A more or less detailed account of our experience with this method has already been published.[17]

We tested the micro method of phosphorus determination in several ways. Parallel determinations were carried out on the same samples of blood and plasma by the gross method described above and by the micro method. For the latter determination about 0.2 gm. of blood or 0.5 gm. of plasma was ashed with 1.5 cc. of acid mixture and the analysis was

[13] Pouget, I., and Chouchak, D., *Bull. Soc. chim. France*, 1909, v, 104; 1911, ix, 649.

[14] Greenwald, *J. Biol. Chem.*, 1915, xxi, 29.

[15] Kober, P. A., and Egerer, G., *J. Am. Chem. Soc.*, 1915, xxxvii, 2373.

[16] Bloor, W. R., *J. Biol. Chem.*, 1915, xxii, 133, 145.

[17] Meigs, E. B., *J. Biol. Chem.*, 1918, xxxvi, 335.

carried further in general as recommended by Bloor.[16] The results were as follows:

Total Phosphorus in Blood and Plasma. Mg. per 100 Gm.

	Micro method.		Gross method.	
		mg.		mg.
			I	18.55
Blood.....		17.94	II	18.51
			Average.....	18.53
			I	13.09
Plasma 1.....		12.80	II	13.70
			Average.....	13.39
	I	12.38		
Plasma 2.....	II	12.07		11.51
	Average.....	12.22		

In other samples of plasma parallel determinations were made of the lipoid phosphorus by the micro method and by the gross method described by Koch and Woods.[18] The results were as follows:

Lipoid Phosphorus in Plasma. Mg. per 100 Gm.

Plasma.	Micro method.	Gross method.
	mg.	mg.
1	7.1	6.8
2	5.0	4.5
3	7.1	6.7
4	7.0	6.5
5	6.4	5.9

We carried out similar parallel determinations on three other samples of plasma, in which the two sets of results did not agree at all; these were among our earliest determinations by the micro method, and we have every reason to believe that the results were incorrect. We do not think it worth while to give the figures for these determinations, and they are mentioned only because this case forms an exception to our rule of giving all the results obtained bearing on any particular point.

It will be seen that the results obtained by the nephelometric method run about 0.4 mg. per 100 gm. higher than do the Koch and Woods results. Following these experiments, we extracted gross samples of plasma with

[18] Koch, W., and Woods, H. S., *J. Biol. Chem.*, 1905–06, i, 203.

alcohol and ether, just as we had done to obtain the Koch and Woods results given above, and then extracted the same samples further, and found that there was additional lipoid in the subsequent extractions. So far as we know, it has been the universal experience of those who have tried to extract lipoid material by alcohol and ether from such large quantities of protein as one gets in gross samples of plasma that the lipoid is not completely extracted in any reasonable time. We agree with Bloor, therefore, that the micro-extraction described by him is decidedly more complete and satisfactory than even very long drawn out gross extractions; and the results given above indicate that our nephelometric results for lipoid phosphorus in plasma are very nearly correct.

Determination of Total Fat.

Fat has been determined in blood and plasma according to the Bloor method and the later modifications suggested by him.[19] This procedure was tried against the well known Babcock method on samples of milk, and was found to give results within 5 per cent of those given by the Babcock method.

Determination of Nitrogen.

In determining nitrogen we have used the Kjeldahl method described by Abderhalden.[20] We encountered no difficulties in following the procedure.

Nature of the Phosphorus Compounds Contained in Normal Plasma.

Abderhalden recognizes the existence of three classes of phosphorus compounds in blood serum: (1) inorganic phosphate; (2) phosphorus combined as lecithin; (3) phosphorus combined as nuclein. He finds in ox serum 0.0037 per cent inorganic phosphorus, 0.0067 per cent lecithin phosphorus, and 0.0006 per cent nuclein phosphorus, making a total of 0.0110 per cent.[21] He has analyzed the serum of a number of other domesticated mammals, and finds very similar figures.

[19] Bloor, *J. Biol. Chem.*, 1914, xvii, 377 ff.; 1915, xxiii, 320.

[20] Abderhalden, Handb. biochem. Arbeitsmethoden, Berlin, 1910, i, 340 ff.

[21] Abderhalden, *Z. physiol. Chem.*, 1897, xxiii, 521; 1898, xxv, 65. The "nuclein" and inorganic phosphorus is given by this author as P_2O_5; and the amount of "lecithin" phosphorus found is not given. The figures which appear above are for elemental phosphorus; those for "nuclein" and inorganic phosphorus are obtained by multiplying Abderhalden's figures by 0.44; those for "lecithin" phosphorus, by multiplying his figures for lecithin by 0.04.

Rona and Takahashi[22] have recently had occasion to determine the different kinds of phosphorus compounds in horse serum. They find, as an average in seven experiments, 0.0005 per cent nuclein phosphorus.

Other work directed more particularly toward the determination of nuclein or protein phosphorus in blood serum makes it seem probable that the figures given by Abderhalden and by Rona and Takahashi are too high. Liebermeister,[23] for instance has endeavored to separate the nucleoprotein from serum and gives figures for the amount of material obtained and for its phosphorus content. He got only 2.5 gm. of nucleoprotein from 15 liters of serum; the phosphorus content of this material was 0.079 per cent. This would indicate only 0.00001 per cent nuclein phosphorus in the whole serum; even if it is supposed that Liebermeister got less than half of the total quantity of nucleoprotein present, his work would still indicate that the nuclein phosphorus content of serum is only a small fraction of that given by Abderhalden and by Rona and Takahashi.

Even the figures of these latter authors are small—about 5 per cent of the total phosphorus contained in serum. Abderhalden gives only a very brief description of his methods; Rona and Takahashi, none at all of theirs; so that one is not able to form a critical judgment as to the correctness of their determination of the nuclein phosphorus.

The evidence so far given, therefore, indicates that there cannot be more than about 0.5 mg. of nuclein phosphorus in 100 gm. of serum, and that there is probably much less than this.

It has been shown by Bürker[24] that adding oxalate to blood prevents the breaking up of the blood platelets, which occurs when it is allowed to clot; when oxalated blood is centrifuged these elements are thrown down at the top of the layer of corpuscles. The plasma from oxalated blood, therefore, differs from serum in that it does not contain the break-down products of these cellular elements. The blood platelets probably contain nucleoprotein;[25]

[22] Rona, P., and Takahashi, D., *Biochem. Z.*, 1913, xlix, 370.

[23] Liebermeister, G., *Beitr. chem. Physiol. u. Path.*, 1906, viii, 439.

[24] Bürker, K., *Arch. ges. Physiol.*, 1904, cii, 88.

[25] Deetjen, H., *Z. physiol. Chem.*, 1909, lxiii, 1. Deetjen has demonstrated that the blood platelets contain nuclei.

and one would, therefore, expect to find less nuclein phosphorus in oxalated plasma than in serum.

In 47 samples of plasma we have made independent determinations of the lipoid, inorganic, and total phosphorus by the methods described above. The results are given in Table I.

The figures in Table I for lipoid, inorganic, and total phosphorus are based on duplicate determinations which agreed within 5 per cent, except as indicated in the footnotes.

Table I shows that the sum of the lipoid and inorganic phosphorus fractions is generally a little lower than the total as determined independently, the difference amounting on the average to 0.26 mg. per 100 gm. of plasma. If the figures for the individual analyses are examined, it will be seen that the individual differences do not vary much from this average. In 37 out of the 47 cases they are not over 0.5 mg. per 100 gm. of plasma; in 9 cases they are over 0.5 mg. and not over 1.0 mg.; and in only 1 case is this difference above 1.0 mg.; namely, 1.7 mg.

In considering the results given in Table I, it is to be remembered that the limits of error for our analyses of total phosphorus in plasma are about 0.5 mg. per 100 gm. of plasma. It is to be remembered also that the analyses from which the figures were taken were, at one time or another, carried out by five different workers, and that as a rule the three analyses for a given sample of plasma were performed by three different workers. Further, each of the methods used for the determination of lipoid, inorganic, and total phosphorus respectively was widely different from either of the others, so that there would be little chance of constant errors in any two of the methods compensating for each other and so masking each other. It seems to us, therefore, that these results indicate fairly clearly that the lipoid and inorganic phosphorus of plasma comprise very nearly all that exists. Whether or not the fact that the sum of our figures for lipoid and inorganic phosphorus is, on the average, 0.26 mg. per 100 gm. lower than the total indicates that plasma contains a very small amount of some other kind of phosphorus, can be better discussed after considering Greenwald's work.

Greenwald has reached conclusions very similar to ours from his recent work on the phosphorus compounds of the blood. His work taken in connection with our own shows that there is no measurable quantity of nuclein phosphorus in plasma.

TABLE I.

Relation between the Sum of the Lipoid and Inorganic Phosphorus Concentrations in Plasma, and the Total as Determined Independently. Mg. per 100 Gm. of Plasma.

No. of cow	Date.	Phosphorus.				
		Lipoid.	Inorganic.	Sum.	Total.	Difference.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*
9	Dec. 15, 1916.....	5.3*	3.9	9.2	9.6	+0.4
	Jan. 17, 1918.....	4.8	6.3	11.1	10.8	−0.3
	Feb. 14, "	4.7	5.3	10.0	10.0	0.0
17	Nov. 1, 1917.....	6.0*	4.1	10.1	10.2	+0.1
19	" 7, 1916.....	8.5	5.9	14.4	13.9	−0.5
	Dec. 22, "	5.7*	5.0	10.7	11.1	+0.4
	Jan. 30, 1917.....	6.4*	6.0	12.4	12.4	0.0
	Mar. 9, "	5.0*	5.6	10.6	11.0	+0.4
	Apr. 9, "	7.3*	4.9	12.2	12.0	−0.2
	May 9, "	8.7	6.8	15.5	15.7	+0.2
	" 22, "	8.1	4.9†	13.0	13.3	+0.3
	June 22, "	8.7	4.6†	13.3	14.0	+0.7
27	Oct. 25, 1916.....	5.6	3.8	9.4	9.7	+0.3
	Dec. 8, "	6.4*	4.9	11.3	11.2	−0.1
	Feb. 12, 1917.....	6.2*	4.1	10.3	10.6	+0.3
33	Oct. 19, 1916.....	7.1*	5.4	12.5	12.7	+0.2
	Nov. 2, "	7.7†	4.6	12.3	13.2	+0.9
	Dec. 4, "	8.3*	5.4†	13.7	14.2†	+0.5
	Feb. 5, 1917.....	8.9*	4.3	13.2	13.4	+0.2
	Oct. 6, "	8.0*	4.2	12.2	12.5†	+0.3
39	" 16, 1916.....	6.4*	5.1	11.5	12.1	+0.6
	" 30, "	6.5	4.6†	11.1	11.2	+0.1
	Nov. 27, "	7.7	5.7	13.4	13.4	0.0
	Jan. 23, 1917.....	6.8*	4.6†	11.4	12.1	+0.7
	Mar. 26, "	6.4	5.3*	11.7	11.5	−0.2
79	Dec. 12, 1916.....	2.0*	6.3	8.3	8.5	+0.2
	Feb. 17, 1917.....	3.1*	6.4*	9.5	9.5†	0.0
80	" 10, "	3.1*	6.1	9.2	9.7‡	+0.5
	Apr. 12, "	2.7*	8.7*	11.4	11.1	−0.3
81	Feb. 20, "	2.6	6.0†	8.6	9.6†	+1.0

TABLE I—*Concluded.*

No. of cow.	Date.	Phosphorus.				
		Lipoid.	Inorganic.	Sum.	Total.	Difference.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*
82	July 11, 1917.....	3.3	5.9*	9.2	10.1§	+0.9
105	Dec. 19, 1916.....	6.3*	4.0	10.3	10.9	+0.6
	Feb. 24, 1917.....	7.2	4.7	11.9	12.6	+0.7
106	Jan. 4, 1918.....	4.9	4.6	9.5	9.5	0.0
107	Dec. 11, 1916.....	7.9*	5.2	13.1	13.4	+0.3
	Mar. 1, 1917.....	8.1*	4.8	12.9	13.2	+0.3
115	Nov. 10, 1916.....	6.1‖	4.8¶	10.9	10.7**	−0.2
	Jan. 4, 1917.....	6.0*	5.3	11.3	11.9	+0.6
	Mar. 3, "	4.8*	4.9	9.7	9.6	−0.1
119	Jan. 11, "	3.5*	6.3†	9.8	10.0†	+0.2
	Mar. 12, "	3.3*	8.8*	12.1	12.0†	−0.1
211	Oct. 16, "	7.5*	4.0	11.5	11.9	+0.4
214	Nov. 20, 1916.....	4.7*	7.8	12.5	12.7	+0.2
	Jan. 18, 1917.....	5.4†	6.1	11.5	13.2	+1.7
	Mar. 16, "	4.9*	6.4	11.3	11.4	+0.1
	Oct. 11, "	5.4*	5.3	10.7	10.8	+0.1
	Nov. 16, "	5.1*	5.1	10.2	9.9*	−0.3

* One determination only.

† See protocol for this animal.

‡ Check 1, 10.2; check 2, 9.3; check 3, 10.5; check 4, 8.8. All four used in calculating average.

§ Check 1, 10.5; check 2, 9.4; check 3, 10.5; all three used in calculating average.

‖ First two determinations discarded on account of lack of agreement. This figure obtained in a single subsequent determination.

¶ Check 1, 4.9; check 2, 4.3; check 3, 4.8; checks 1 and 3 used in calculating average.

** Check 1, 11.6; check 2, 10.5; check 3, 10.9; check 4, 10.6; checks 2, 3, and 4 used in calculating average.

Greenwald extracts the acid-soluble phosphorus from serum with a mixture of picric and acetic acids and he has shown that none of the lipoid phosphorus is extracted from his material by this reagent.[26] In a later article he considers the question whether

[26] Greenwald, *J. Biol. Chem.*, 1913, xiv, 369.

the phosphorus extracted by the picric-acetic acid is all strictly inorganic or whether there is mixed with it a small amount of soluble organic phosphorus.[11]

He divides his samples of serum into four portions, A, B, C, and D. In A, B, and C the protein and phosphatide are precipitated, and the acid-soluble phosphorus is extracted with picric-acetic acid mixture; in D the inorganic phosphorus is precipitated directly by magnesia mixture and subsequently determined. The picric-acetic acid extract from A is ashed with acid mixture and its total phosphorus determined; in the extracts from B and C the inorganic phosphorus is precipitated (by two different methods) and determined. The results from B, C, and D agree fairly closely with each other on the average, and are all about 0.3 mg. per 100 gm. of serum lower than those from A. Greenwald considers that in A he gets the total acid-soluble phosphorus; and in B, C, and D, the inorganic phosphorus; and he concludes that serum contains a small amount of acid-soluble phosphorus which is not strictly inorganic. To us it seems that his results might equally well be interpreted by supposing that the methods of analysis adopted in the cases of B, C, and D all give results which are a little low. It is to be noted that in all three of these analyses, and particularly in B and C, the phosphorus (small in amount) is precipitated from a large volume of fluid; this is presumably not the case in A; and it seems not unlikely that this circumstance alone might account for the lower results in B, C, and D.

However this may be, it is evident that the method used in D does not get all of the acid-soluble phosphorus from serum, the amount not accounted for being about 0.3 mg. per 100 gm. of serum. The method used in D is essentially the same as our routine method for inorganic phosphate; therefore, in order to calculate the total acid-soluble phosphorus in our samples of plasma from our routine figures for inorganic phosphate, one would have to add 0.3 mg. per 100 gm. to each of our results. This would slightly more than cover the average difference between our figures for total plasma phosphorus and those for the sum of the lipoid and inorganic; the difference in question was 0.26 mg. per 100 gm. In other words, our results show that our samples of plasma contained only lipoid and acid-soluble phos-

phorus; the difference between the sum of these quantities and the total as determined independently is only 0.04 mg. per 100 gm. of plasma. It is evident that there can be no nuclein phosphorus in either the lipoid or acid-soluble fractions, and it follows that plasma contains no nuclein phosphorus.

Greenwald has reported an experiment[27] which indicates that serum contains no nuclein phosphorus. He precipitates and extracts serum with acetone, reextracts the precipitate with alcohol and ether, and finally with picrohydrochloric acid mixture; he finds only a negligible quantity of phosphorus in the residue thus extracted. No figures are given, however, from which one could judge the completeness of the demonstration that nuclein phosphorus is absent, and we feel justified in publishing our results as a confirmation and extension of his conclusion.[28] There remains the question whether the small discrepancy between the acid-soluble phosphorus of plasma and the inorganic as determined by the direct precipitation of magnesium ammonium phosphate without previous protein precipitation is or is not merely the result of the failure of the latter method to account for all of the inorganic phosphorus. We are inclined to think that it is. It has been pointed out above that Greenwald's results bearing on this question do not seem conclusive; and we think that weight should be given to the fact that the "inorganic phosphate" of plasma as determined by the Embden method averages a little higher than that obtained by our routine method (see above under Methods). We shall not give here a detailed account of the former Method. It is only necessary to say that Embden and his collaborators were satisfied, after testing it in various

[27] Greenwald, *Am. J. Med. Sc.*, 1914, cxlvii, 226.

[28] We fear, however, that a table published by him (*J. Biol. Chem.*, 1915, xxi, 32) may cause us to be accused of simply repeating his experiments. At first sight, this table does appear to be exactly similar to our Table I, but in the text of the article (pp. 30, 31) one finds that the figures for "lipoid phosphorus" given in Greenwald's table represent the total phosphorus left in the residue from serum extracted with picric-acetic acid mixture. We do not object to this method of estimating the lipoid phosphorus of serum, but we wish to point out that Greenwald's table has no value as a demonstration that serum contains only lipoid and acid-soluble phosphorus; it is only through the previous proof of this proposition that his figures for lipoid phosphorus attain their value.

ways, that it gave correct results for the inorganic phosphate of muscle juice. Their results were lower than most of the previous figures given for the inorganic phosphorus of muscle and muscle juice. There can be no doubt that muscle juice contains considerable amounts of labile organic phosphorus compounds, and a method capable of separating the strictly inorganic phosphorus from these would be unlikely to give high results for the inorganic phosphate of blood plasma.

If our conclusions are correct, it follows that the plasma does not serve as a vehicle for the transport of nucleins or phosphorized proteins, and that these compounds must be synthesized *in situ* by the cells in which they are found. It follows also that if one knows the total phosphorus of a sample of plasma, and either the lipoid or inorganic phosphorus, one can readily calculate the amount of the missing fraction.

Precursor in the Blood Plasma of Milk Phosphorus and Milk Fat.

Most of the work directed toward discovering in the blood the precursors of the various constituents of milk has been devoted to the question of the precursor of lactose. It has been established that the glucose of the blood is the precursor of lactose; evidence for this view has been obtained by two different methods of investigation. One of these is to remove the mammary gland from the body, to perfuse it, and to study the concentration of the materials in question in the perfusion fluid before and after it has been sent through the gland, as well as in the fluid secreted by the gland. The other method is to obtain samples of blood from one of the mammary veins and from some other superficial vein, say the jugular, in the intact animal, and to compare the concentrations of the materials in question in such samples of blood.

The second method seems to us the more promising, because when using it one is working with a gland which is functioning under much more nearly normal conditions, and there are many reasons for thinking that the functioning of the mammary gland is easily disturbed.

There are, however, objections to the second method, which must be considered. One of these is that, in a single passage of

the blood through the mammary gland, its composition would probably be so little changed that the change could not be detected by the biochemical methods which are at present at our disposal. The most effective answer to this objection lies in the results that have been obtained by the method by other investigators as well as by us. These will be reported and discussed later. Another objection is that blood obtained from the jugular vein cannot be regarded as strictly equivalent to the blood carried to the mammary gland by its artery; this objection may be answered as follows.

The blood pumped by the left ventricle of the heart into the aorta is presumably thoroughly mixed, so that all parts of the body supplied by the aorta receive identical samples of blood. It may, therefore, be presumed that the blood supplied to the tissues drained by the jugular vein is the same as that supplied to the mammary gland. The tissues drained by the jugular vein are, for the most part, muscles, and the changes brought about by muscles in the phosphorus or sugar content of blood passing through them would be negligibly small in comparison to that brought about by the mammary gland. The blood of the jugular vein would, therefore, for all practical purposes be identical with the arterial blood going to the tissues drained by that vein, and hence also with the arterial blood going to the mammary gland.

The blood which has passed through the cow's mammary gland is to a large extent carried away by the abdominal subcutaneous veins or milk veins.[29] These veins run forward from the udder on the abdomen immediately under the skin; in milking cows they are usually extremely large and prominent, and blood may be obtained from them by means of a trochar and cannula rather more easily than from the jugular vein.

Our experimental procedure has consisted in obtaining samples of blood from the milk veins and from the jugular veins of milking and dry cows, as nearly simultaneously as practicable, and in analyzing the blood and plasma from such samples for total, lipoid, and inorganic phosphorus. Five such experiments have

[29] Chauveau, A., The comparative anatomy of the domesticated animals, New York, 2nd edition, 1905, 711, 998–999. Sisson, S., The anatomy of the domesticated animals, Philadelphia and London, 2nd edition, 1914, 609, 721.

been carried out on milking cows and three on dry cows. Of these eight experiments, the results of five are reported in detail in the protocols of Experiments 1 to 5.

In Experiment 1 the chemical analyses ran smoothly. The differences between checks are less than 2 per cent throughout, and the lipoid and inorganic fractions in both jugular and mammary plasma add up to very nearly the amount of the totals as determined independently. The result seems at first sight rather surprising. Apparently the only change brought about in the plasma by its passage through the mammary gland is an increase in the amount of inorganic phosphate contained in it, amounting to 0.65 mg. per 100 gm. of plasma. As there is no change in the lipoid phosphorus, the increase in inorganic shows itself as an increase in the total, so that the mammary plasma contains more phosphorus than does the jugular.

Such a condition cannot, of course, be usual in milking cows. The mammary gland of a milking cow must, on the whole, be taking phosphorus from the blood instead of discharging phosphorus into it, and Experiment 1 must represent abnormal or unusual conditions. The subject of this experiment had been greatly disturbed by the experimental procedure (see protocol), and we, therefore, carried out further experiments in which our methods of avoiding disturbance to the subjects were gradually improved. The first two of these gave results essentially similar to those of Experiment 1, and are not reported in detail here. In the first of them the cow was almost as much disturbed before the collection of the mammary blood as in Experiment 1. In the second, she was decidedly less disturbed than in Experiment 1, and there was some indication that the lipoid phosphorus in the mammary plasma was decreased. The decrease was small, however, in comparison to the increase in the inorganic phosphorus, and our checks did not agree closely enough to satisfy us that it might not have been the result of experimental error. In this experiment we compared the total phosphorus content of the jugular and mammary blood, as well as that of the plasma, and found an increase in the total phosphorus of the mammary blood closely corresponding to the increase found in the mammary plasma.

In Experiments 2 and 3 our methods of avoiding disturbance to the subjects before and during the taking of the mammary samples were much improved. These experiments, like the three others, indicate that, at the time they were being carried out, the mammary gland was discharging inorganic phosphate into the blood stream. But they differ from them in indicating that at the same time the gland was taking up lipoid phosphorus from the blood. In Experiment 2 enough lipoid phosphorus was taken up to compensate for the discharge of inorganic phosphate into the blood; in Experiment 3 the discharge of inorganic phosphate was decidedly larger than in any of the others and the amount of lipoid phosphorus taken up was not enough to compensate for it. But the lipoid phosphorus taken up was nevertheless larger than in the case of Experiment 2—somewhere between 0.55 and 0.74 mg. per 100 gm. of plasma.

The experiments taken together indicate that in milking cows the mammary gland is continually discharging inorganic phosphate into the blood; that, when the cow is undisturbed, the gland takes lipoid phosphorus from the blood; but that this latter process is interfered with by even comparatively slight disturbance. It seems to us that these results can best be interpreted by supposing that the gland gets from the blood both the fat and phosphorus which it requires for milk secretion in the form of phosphatide—either lecithin or some related body.

Lecithin contains about one part by weight of phosphorus to eighteen of fat; other phosphatides contain a still larger amount of phosphorus in proportion to the fat. Milk, on the other hand, contains one part of phosphorus to about 50 parts of fat. It is obvious, therefore, that if the mammary gland gets from the blood enough phosphatide to supply the milk with fat, it must get with it more phosphorus than is required for the milk, and the excess must be returned to the blood. These considerations explain the back-flow of inorganic phosphate from the gland to the blood which occurred in all of the experiments.

It will be well to continue the discussion of these experiments by referring to the work of Kaufmann and Magne[30] who compared the sugar content of the jugular and mammary blood of milking

[30] Kaufmann, M., and Magne, H., *Compt. rend. Acad.*, 1906, cxliii, 779.

and dry cows. Our experience has been, in many respects, parallel to theirs.

These authors have investigated the mammary secretion of lactose by comparing the sugar content in samples of blood obtained nearly simultaneously from the mammary and jugular veins. They found that in milking cows the mammary blood always contained less sugar than the jugular. Their experiments were carried out on different cows giving different amounts of milk and at different periods after milking, etc., so that the averages of their figures are not very significant. But their results in round numbers may be indicated by saying that the jugular blood generally contained about 0.08 per cent of sugar while the mammary blood contained only 0.07 per cent; each 100 cc. of blood in passing through the gland gave up to it about 0.01 gm. of sugar Their cows, as nearly as can be judged from their description, gave about 10 liters of milk daily; and, by using these figures and reckoning the amount of lactose in the milk at 5 per cent, a rough calculation can be made of how much blood was passing through the mammary gland per minute in their experiments.[31] 10 liters of milk with a 5 per cent lactose content would contain 500 gm. of lactose; in other words the cows used by Kaufmann and Magne were secreting about 500 gm. of lactose daily. They were secreting, therefore, $500 \div (24 \times 60)$ or about 0.35 gm. of lactose per minute. And, if each 100 gm. of blood passing through the mammary gland yielded to it 0.01 gm. of sugar, it would require 100×35 or 3.5 liters of blood to yield 0.35 gm. of lactose; that is, according to Kaufmann and Magne's figures, about 3.5 liters of blood were passing through the udders of their cows per minute. It is realized that this estimate is very rough; no doubt the rate at which the blood passes through the mammary gland varies enormously in different cows and under different physiological conditions. But to have even a rough idea of this rate is desirable, in order to see to what extent the

[31] As far as we know, the experimental evidence is all in favor of the view that in lactating animals, milk secretion is going on all the time. See Roehrig, A., *Virchows Arch. path. Anat.*, 1876, lxvii, 119. Foà, C., *Arch. Fisiol.*, 1912, x, 402. See also Gaines, W. L., *Am. J. Physiol.*, 1915, xxxviii, 303. Our own results and those of Kaufmann and Magne still further confirm this view.

results of our experiments would account for the secretion of milk fat.

The cows used in Experiments 2 and 3 were giving each about 10 liters of milk daily and about 400 gm. of fat. Their glands, therefore, would take from the blood about $400 \div (24 \times 60)$ or 280 mg. of fat per minute. Supposing that 3 liters of blood were passing through the gland per minute, this would mean that from each 100 cc. of blood passing through $280 \div 30$ or 9 mg. of fat in the form of phosphatide would be taken. As the proportion of fat to phosphorus in phosphatide is about 18: 1, this would mean that $9 \div 18$ or 0.5 mg. of lipoid phosphorus would be taken from each 100 cc. of blood passing through the gland. This figure corresponds satisfactorily with those which have been actually found. While we realize very fully the possible inaccuracy of many of the assumptions on which the above calculation is based, we still feel that the agreement between the expected and the experimental figures for the differences in lipoid phosphorus concentration as between jugular and mammary plasma helps to confirm the view that milk fat is derived from plasma phosphatide.

It would be satisfactory if, in such experiments as those described above, determinations of total fat in the plasma could be made along with the phosphorus determinations to demonstrate that the decrease in total fat in the mammary plasma is only sufficient to cover the decrease in phosphatide. In some of the experiments fat determinations were made according to the nephelometric method described by Bloor (see above) and it was found that the plasma contained about 0.8 per cent of total fat. The expected decrease would be 0.009 per cent, only a little more than one hundredth part of the total, and we fear that none of the fat methods with which we are familiar could demonstrate such small proportional differences.

We did, however, determine the total fat in the jugular and mammary plasma in Experiment 1. The results indicated, as far as they went, that the jugular and mammary plasma contained the same amounts of fat, but the differences between the checks were very much larger than the difference that might be expected between the phosphorus contents of the two samples of plasma as the result of the taking up of fat by the mammary gland.

We endeavored to control our experiments as Kaufmann and Magne did theirs by comparing samples of jugular and mammary blood from cows that were not milking. The results are given in the protocols, Experiments 4 and 5. Considerably to our surprise, these cows also were apparently discharging inorganic phosphate from the mammary gland into the blood, and at about the same rate, as far as could be judged from the figures, as the milking cows. Neither cow appeared to be taking up lipoid phosphorus from the blood into the gland. Cow 106, Experiment 4, had been dry for only a few days, and still had a good deal of milk in her udder; Cow 115, Experiment 5, had never had a calf, and was probably not pregnant at the time the experiment was carried out.

These results made us think, at first, that our earlier results on the milking cows might have had nothing to do with milk secretion; and they caused us to look very carefully into the technique of our experiments and into the physiology of the mammary gland in "dry" cows.

As was said above, the milk vein in a milking cow is very large and close under the skin. When the animal is standing, it lies considerably lower than the jugular vein; and partly for this reason, partly, perhaps, because the blood flow through the active gland is very free, one can collect blood sufficiently rapidly from it through quite a small cannula. We found that in order to collect blood from the mammary vein, it was not necessary to fasten the cow in any special way at all. She was simply allowed to stand with her head in the stanchion as she does at all times when she is standing in her stall. One of the assistants held his arm in front of her hind legs while the mammary cannula was being inserted to avoid any possibility of effective kicking; but, in Experiments 2 and 3, in which the reduction of lipoid phosphorus in the mammary plasma was demonstrated, this precaution turned out to be unnecessary, for the cows made no attempt to kick, and, indeed, seemed hardly to notice the insertion of the trochar and cannula at all.

To collect blood from the jugular vein is not so easy. It is necessary, in the first place, to hold the cow's head so that her neck shall be more or less extended, and this procedure always causes some uneasiness. As the vein lies deeper under the skin

than the milk vein, a larger cannula is necessary, and it is generally not quite so easy to insert it. But the process is not a complicated one. Only a few minutes elapsed in Experiments 2 and 3 between the end of the collection of the mammary blood and the beginning of the collection of the jugular blood.

The result of our first three[32] experiments indicated that it takes only a small disturbance to interfere with the taking up of phosphorus from the blood by the mammary gland. It was thought advisable, therefore, to avoid disturbance as far as possible before and during the collection of the mammary sample, and for this reason the whole procedure necessary for the collection of the jugular sample was left, in Experiments 2 and 3, until after the mammary sample had been collected. It was realized that this course introduced the objection that the cow was somewhat disturbed between the collections of the two samples of blood that were to be compared with each other, but it was not thought that the disturbance would be sufficient to alter materially the phosphorus content either of the blood as a whole, or of the blood coming from the jugular vein. Further, we hoped to control this point completely by our experiments on the dry cows. But our experiments on the dry cows do not furnish a complete control in regard to this point. As far as the increase in inorganic phosphate in the mammary plasma is concerned it occurs in the dry cows as well as in the milking ones, and to a surprisingly similar extent. The decrease in the lipoid phosphorus of the mammary plasma does not occur in the dry cows. The experiments on the dry cows were carried out later than Experiments 2 and 3, and we tried to make them similar to these in every respect, so that, as far as the lipoid phosphorus is concerned, they do control Experiments 2 and 3 to a certain extent. But they do not do so completely, because the milk veins in dry cows are smaller than in milking cows—in heifers that have never had calves they are very much smaller. For this reason it is more difficult to insert a trochar and cannula in the milk vein of a dry cow, and the animals used in Experiments 4 and 5 showed more sign of disturbance when the cannula was in-

[32] Between Experiments 1 and 2, two others were carried out which had the same results as Experiment 1. It has not been thought worth while to report them in detail.

serted in their milk veins than did the animals used in Experiments 2 and 3. Both the cows used in Experiments 4 and 5 made attempts to kick. It was thought desirable, therefore, to control the experiments in another way.

The method chosen was to obtain two samples of jugular blood, one with as little disturbance as possible to the subject, and the other after the same amount of disturbance as occurred in Experiments 2 and 3. For this purpose a cow was chosen which had been dry for some time. The details of the experimental procedure and the results are given in the protocols under Experiment 6. It will be noted that there is no significant difference between the two samples of blood and plasma with respect either to lipoid or inorganic phosphorus; and the experiment may, therefore, be taken to indicate that the differences in the phosphorus content of jugular and mammary blood and plasma observed by us in Experiments 1 to 5 were not due to any change in the whole body of blood brought about by the disturbance of the subject which occurred between the collections of the mammary and of the jugular samples.

We have pointed out above that the tissues drained by the jugular vein are for the most part muscles, and that the phosphorus metabolism of muscle would be too small to affect the results of such experiments as we have been discussing. But we feel that it is worth while to consider the phosphorus metabolism of muscle more fully at this point, both on account of its bearing on the results of these experiments, and on account of its own intrinsic importance.

It has been shown by a number of workers that muscular exercise in intact animals and human beings causes an increase in the phosphate excreted with the urine,[33] and that the activity of striated muscle results in an increase in the inorganic phosphate contained within it.[34] These results may be taken to show that in active muscles inorganic phosphate is produced from some

[33] Flint, A., Jr., *N. Y. Med. J.*, 1871, xiii, 609. Klug, F., and Olsavszky, V., *Arch. ges. Physiol.*, 1893, liv, 21. For further references on this subject see Forbes, E. B., and Keith, M. H., A review of the literature of phosphorus compounds in animal metabolism, *Ohio Agric. Exp. Sta., Technical Bull. 5*, 1914, 464 ff.

[34] Macleod, J. J. R., *Z. physiol. Chem.*, 1899, xxviii, 535.

organic phosphorus compound, and that the inorganic phosphate so formed tends to escape to the blood and to be excreted in the urine. But Macleod has shown that the muscles of animals which have been put through a period of vigorous exercise contain more total phosphorus than do unexercised muscles.[34] As the exercised muscles contain more total phosphorus in spite of the fact that, during exercise, muscles tend to give up inorganic phosphate to the blood, they must have taken up organic phosphorus from the blood. And, as the blood plasma contains only lipoid and inorganic phosphorus, the organic phosphorus must have been taken up in the form of phosphatide.

It would seem, therefore, that the phosphorus and fat metabolism of the mammary gland and of striated muscle are roughly parallel. Both tissues receive their fat and phosphorus from the blood in the form of phosphatide; both convert the phosphatide into fat and inorganic phosphate; they use the fat, in the one case, to supply the fat of milk, in the other, to burn and supply energy; and they return the inorganic phosphate largely or wholly to the blood. It is impossible, therefore, to explain the differences which have been observed by us in the lipoid and inorganic phosphorus content of jugular and mammary blood as the result of phosphorus metabolism carried on in the muscles drained by the jugular vein. Muscles take lipoid phosphorus from the blood passing through them and discharge inorganic phosphorus into it; and the results of our experiments show, as was to have been expected, that these processes go on much more rapidly in the case of the mammary gland than in the case of the muscles.

It is justifiable to conclude, then, that the differences in the phosphorus content of jugular and mammary plasma observed by us are the result of the metabolic activity of the mammary gland. Two aspects of the results remain to be discussed; namely, the fact that the taking up of lipoid phosphorus by the gland was never more than enough to balance its discharge of inorganic phosphorus, and the fact that such large amounts of inorganic phosphorus were discharged into the blood by the glands of the non-milking cows.

We believe that the first of these circumstances is to be explained partly by the supposition that even in our most successful experiments the subjects were enough disturbed by the procedure

to interfere to some extent with the taking up of phosphatide by the mammary gland. But we believe also that in most of the experiments the procedure caused a more than normally rapid discharge of inorganic phosphate from the gland to the blood. In all except Experiment 2 the mammary gland was more or less physically agitated. It seems not improbable *a priori* that physical agitation of the mammary gland would accelerate the discharge of phosphate from its cells to the blood, and the experiments furnish evidence indicating that this is the case.

The least difference in inorganic phosphate content of jugular and mammary blood that we obtained was in the case of Cow 211, Experiment 2. In this case the subject's udder was not disturbed at all either by milking or by any attempt on her part to kick. The greatest difference was obtained in the case of Cow 17, Experiment 3, where the calf was allowed to suck before and during the collection of the mammary blood sample, and spent much of the time when it was supposed to be sucking in butting the udder with its nose. In the other experiments, where the results were intermediate, the udder was physically agitated, though decidedly less than in Experiment 3, sometimes by having the cow milked by an assistant during the collection of the mammary sample, sometimes by her own attempts to kick.

We believe that the discharge of inorganic phosphate from the mammary gland cells to the blood in the non-milking cows is to be explained by supposing that these cells remain to some extent active for a long time after a cow has become dry, and become active in heifers before they have become pregnant for the first time. We should feel some timidity about basing this statement on the results of our blood analyses alone, but they are supported by so much independent evidence that there can be no doubt of their truth. Several cc. of milk were readily obtained from the udder of each of the dry cows used in the experiments described above, and from another cow, selected for examination because she had been dry for some time. These cows had not been milked for 6 days, 25 days, and nearly 4 months respectively. A few cc. of milk were also obtained from the udder of Cow 115, a heifer about 3 years old which had never had a calf and was probably not pregnant, though she had been bred unsuccessfully a number of times. As Cow 115 might possibly have been pregnant from

some of the later services, another heifer was selected for examination. This animal was 15 months old and had never been bred at all. A few drops of milk were obtained without much difficulty from her udder. Hill[35] has recently reported the case of a virgin kid, which yielded 120 cc. of milk when it was about 4 months old. It was milked at intervals for the next 3 months, 1,900 cc. being obtained altogether. The milk was analyzed and found to be normal in every way. A somewhat similar case has been reported by Pfaundler,[36] and Schein[37] has elaborated a theory of continuous milk secretion.

The difference between the concentrations of inorganic phosphate in the jugular and mammary plasma of non-milking cows is about the same as in the milking cows. We believe that this circumstance is connected with the fact that the blood flows very much less rapidly through the glands of non-milking animals. We have had occasion to make a more or less careful examination of the milk veins of mature dry and milking cows, and of young farrow heifers. In the milking cows this structure is very large, and, in the heifers, quite small; it probably carries not more than one-tenth the amount of blood in the latter case, and perhaps even much less than this. In the dry mature cows that we have examined, the milk vein has perhaps nearly the same capacity as in the milking ones, but it gives the impression of carrying very much less blood. It feels less distended, and blood flows decidedly slower from a cannula inserted in it. It seems not improbable that the activity of the mammary gland cells is dependent on the amount of blood flowing through the gland as much as on any other single factor.[38]

Many of the features of fat and phosphorus secretion by the mammary gland, as indicated by our results, are closely paralleled by what is already known regarding the secretion of carbohydrate. It has been observed for instance by several investigators that animals in the early stages of lactation or just before parturition are likely to exhibit lactosuria. Thus von Noorden[39]

[35] Hill, R. L., *J. Biol. Chem.*, 1918, xxxiii, 391.

[36] Pfaundler, M., *Z. Kinderh.*, 1912, iii, 191.

[37] Schein, M., *Wien. klin. Woch.*, 1910, xxiii, 1337.

[38] The same conclusion, though from very different evidence, has been reached by Roehrig.[31]

[39] von Noorden, H., *Arch. physiol.*, 1893, 385.

found that when glucose was given *per os* to a newly lactating goat, lactose appeared in the urine. Porcher has repeated this experiment on a dog and obtained similar results.[40] And Kaufmann and Magne[30] found that one of the cows experimented on by them exhibited lactosuria 24 hours before she calved and for 3 days thereafter. They obtained blood samples from the jugular and mammary veins of this cow 24 hours before she calved and found that the mammary blood contained considerably less sugar than the jugular, though at this time the cow was presumably giving no milk. They are extremely brief in giving their methods of analysis and their experimental results; but, as far as can be judged from what they say, the decrease in the figure obtained by them for the sugar content of mammary blood would be, at least in part, accounted for by supposing that some of the glucose was taken out of the blood by the mammary gland and that lactose was substituted for it.

There is thus evidence that the lactose which the mammary gland cells manufacture from glucose may, under certain circumstances, be partly returned to the blood, just as is a part of the phosphate which they manufacture from phosphatide; and also that before parturition, when the gland is not actively secreting milk, its cells may, nevertheless, be taking up glucose from the blood, converting it to lactose, and returning this to the blood, just as they may be taking up phosphatide, and returning the phosphate, and presumably the fat also, to the blood.

In a number of experiments on the comparison of jugular and mammary blood, it was found that the jugular blood contained a greater volume of corpuscles than the mammary (see protocols). The differences varied greatly, but were always in favor of the jugular blood, if they occurred at all. In Experiment 3 on Cow 17 the jugular blood contained 31.4 volumes per cent of corpuscles, whereas the mammary contained only 28.2 volumes per cent, so that the corpuscles in the mammary blood were decreased by more than 10 per cent of their volume in the jugular blood. It is unlikely that any osmotic changes in the plasma brought about by the taking up of water, salts, or sugar by the mammary gland could produce so large a shrinkage in the volume of the corpuscles; and it is probable, therefore, that the difference is to be

[40] Porcher, C., *Arch. internat. physiol.*, 1909, viii, 356 ff.

explained as a difference in the number of corpuscles in the two samples of blood. An examination of the figures for total phosphorus in blood and plasma in this experiment supports this view. In the case of the whole blood, the jugular sample contains more total phosphorus than the mammary, while these relations are reversed in the case of the two samples of plasma. As the corpuscles contain considerably more phosphorus per unit of volume than the plasma, this circumstance is very well explained by supposing that in this experiment the jugular blood contained a larger number of corpuscles than the mammary, and that the phosphorus contained in these extra corpuscles was more than enough to offset the lesser phosphorus content of the jugular plasma.

It would seem, therefore, that, under the conditions of our experiments the jugular blood is likely to contain a rather variably larger number of corpuscles than the mammary. If this phenomenon showed more regularity, it might conceivably be taken to mean that corpuscles had a tendency to become engaged and held in the capillaries of the mammary gland, and there broken up, while the products of their decomposition are used for milk secretion. But the greatest difference between jugular and mammary corpuscles was in the case of Cow 115, which had never had a calf, and in which there was no sign of the taking up of lipoid phosphorus by the mammary gland; while in the case of Cow 211, in which secretion was going on actively, the difference was small. It seems to us more likely, therefore, that the phenomenon is some unforeseen effect of the experimental procedure; possibly the movements of the head, which always occur as the result of the collection of the jugular sample, result in the washing out from the capillaries of the muscles drained by the jugular vein of a varying small amount of corpuscles which have collected there during the preceding period of greater quiescence.

It might be expected that, if plasma phosphatide is the precursor of milk fat, there would be some tendency to parallelism between the concentration of phosphatide in a cow's plasma at any given time and the percentage of fat in the milk secreted at the same time. With this idea in mind we have compared the phosphatide concentrations in a number of our samples of plasma with the fat in the milk yielded by the subjects of the ex-

periments at about the time the blood samples were obtained. The results have shown it to be a rough general rule that cows with a high percentage of plasma phosphatide are likely to yield milk rich in fat, but there are many individual exceptions. The most that can be said is that, while the phosphatide concentration in the plasma is probably one of the factors which influence the fat content of milk, there are other factors which often mask its influence.

The literature on the immediate precursor of milk fat is not large. The evidence which bears most directly on the question is contained in the experiments of Foà.[41] This author perfused the isolated mammary glands of sheep with various fluids, and showed quite conclusively that milk secretion may still go on in the isolated gland. His results indicate also that milk fat may be derived from triglycerides in the blood.

He found that when he perfused Ringer's solution in which olive oil or triolein was emulsified, through the vessels of the gland, he obtained a fluid from the duct which contained fat. That the olive oil and triolein emulsions had not simply filtered through the gland cells into the ducts is shown by the facts that the fat in the fluid obtained from the duct was in the form of globules with the microscopic appearance of those normally seen in milk, and that it had a lower iodine number than the olive oil and triolein mixed with the perfusion fluid. That the fat in the fluid issuing from the duct was not simply fat which had been present in the gland cells before the beginning of the experiment and had been washed out into the ducts by the perfusion fluid is indicated by the fact that when Ringer's solution without fat was perfused through the vessels, there issued from the duct a fluid which contained no fat.

We think that the olive oil and triolein may have entered the gland cells from the perfusion fluid, and that they may have been partially changed within the cells to fats with lower iodine numbers; but we do not think that this proves that triglycerides enter the gland cells from the blood under normal conditions. In the three experiments of Foà which bear on the point the gland became edematous quite early, and in all of them the collection of the fluid issuing from the duct, which was afterward analyzed, was continued after the edema had been noted. What-

[41] Foà, *Arch. Fisiol.*, 1912, x, 402.

ever theory one may hold regarding the nature of cell surfaces, it seems reasonable to suppose that a perfusion fluid, which produced edema in the mammary gland in between 1 and 2 hours, would alter the surfaces of the cells directly presented to it before the edema became perceptible. Alterations in cell surfaces brought about by abnormal influences are almost always in the direction of increased permeability.

The cell surfaces of the glands used by Foà were, therefore, probably abnormally permeable, and the fact that triglycerides passed through them would not prove that they could pass through under normal conditions. But it is not necessary to suppose that an alteration in the surfaces of the mammary cells would immediately suspend all their activities. Urano[42] has shown that striated muscle cells may still contract long after the permeability of their surfaces has been abnormally increased, and one of us[43] has shown that a similar retention of function may occur in clam's muscle after it has been for 40 hours in pure cane sugar solution and after most of the salts that are normally present have diffused out and become substituted by sugar. It would not be surprising, therefore, if it should turn out that the mammary cells still retained the power of acting on combinations of glycerol and fatty acids after their surfaces had been so altered as to permit the passage of materials to which they are normally impermeable.

We think that our results indicate, though they cannot be said to prove that milk fat is not normally derived from the triglycerides of the blood. In two of our experiments the taking up of phosphatide from the blood by the gland cells is directly demonstrated, and the rate at which this process occurs is, as far as can be judged from the data at hand, sufficient to account for the daily production of milk fat on the supposition that it all comes from the phosphatide of the blood. In all of the experiments there is demonstrated a considerable back-flow of inorganic phosphate from the gland to the blood, which is easy to understand on the supposition that it represents the excess phosphate brought into the gland cells with the phosphatide necessary for milk fat secretion, but is left as a very puzzling fact, if we must sup-

[42] Urano, F., *Z. Biol.*, 1908, l, 212. See also Fahr, G., *ibid.*, 1909, lii, 72.
[43] Meigs, E. B., *J. Biol. Chem.*, 1915, xxii, 496–497.

pose that milk fat is derived both from phosphatide and from triglycerides which contain no phosphorus.

Finally it seems to us an improbable supposition that the mammary gland derives its fat both from phosphatides and from triglycerides in the blood. It is our belief that phosphatide furnishes a form in which fatty acids may readily pass through cell surfaces, and we are inclined to think that the very rapid excretion of fat accomplished by the mammary gland is made possible by this circumstance.

Jordan and a number of coworkers[44] have shown that reducing the amount of phosphorus in a cow's rations sometimes reduces not only the percentage but also the absolute amount of the fat secreted with the milk. This result does not always occur, but in some cases it is quite marked and persists for some time. It is not to be expected that this result would occur with complete regularity. It has been shown repeatedly[45] that a milking cow is capable of taking large amounts of calcium and phosphorus from her bones to supply any deficiency in these elements that may exist in her rations. It is quite possible, therefore, that, unless a cow's phosphorus stores were already more or less depleted, a deficiency of phosphorus in the diet would not show itself in any disturbance of fat metabolism. But that a deficiency in the phosphorus intake results even occasionally in a decrease in the excretion of milk fat is fairly strong confirmatory evidence for the view that the phosphatide of the blood plasma is the precursor of this product.

The following is a résumé of the conclusions drawn from our experimental results. The fat and phosphorus which are excreted with milk come from some phosphatide body or bodies in the blood. This material is converted in the mammary gland cells to inorganic phosphate and neutral fat; it yields two and a half or more parts of phosphorus to forty-five of fat; and, as milk contains only about one part of phosphorus to forty-five of fat, some

[44] Jordan, W. H., Hart, E. B., and Patten, A. J., *Am. J. Physiol.*, 1906, xvi, 268.

[45] Hart, E. B., McCollum, E. V., and Humphrey, G. C., *Am. J. Physiol.*, 1909, xxiv, 100; Forbes, E. B., Beegle, F. M., Fritz, C. M., Morgan, L. E., and Rhue, S. N., *Ohio Agric. Exp. Sta., Bull. 295*, 1916; *ibid., Bull. 308*, 1917.

60 per cent of the phosphorus which enters the gland cells with the phosphatide precursor of milk fat must be returned to the blood as inorganic phosphate. This back-flow of inorganic phosphate from the mammary gland to the blood is not necessarily synchronous with the taking up of phosphatide by the gland. The gland cells are at all times loaded with excess phosphate, and the escape of this excess to the blood may continue for some time after the taking up of phosphatide has ceased. The escape of excess phosphatide to the blood is probably accelerated by any physical manipulation or agitation of the mammary gland. The taking up of phosphatide by a cow's gland is stopped if she is subjected to an even slight psychological disturbance.

The mammary gland cells remain to some extent active after a cow has ceased to be milked, and they become active in heifers even before they have become pregnant for the first time.

Concentrations of Calcium and Phosphorus in the Blood and Their Relation to Milk Secretion.

Calcium and phosphorus are present in rather small concentration in the blood, but nevertheless participate actively in milk secretion. In the case of a cow giving 20 liters of milk per day, and having about 25 liters of blood in her body altogether, the total amount of each of these constituents contained in the whole body of blood at any time is much less than that excreted with the daily yield of milk. We must think, therefore, of these two elements as being rapidly taken out of the blood by the mammary gland and equally rapidly poured into it from other sources; the maintenance of the concentration of each of them in the blood will depend on the equalization of these two processes.

It is obvious that milk secretion must, to some extent, depend on the maintenance of a certain amount of each of these materials in the blood. If either of them should be exhausted in the blood, the secretion of milk would probably cease.

Further, the calcium and phosphorus present in the blood must come ultimately from the food, and their concentration in the blood must therefore, depend, either immediately or remotely on the amount of them supplied with the food. A knowledge of how the concentrations of these materials behave in the blood under

various circumstances can hardly fail to throw light on the various problems connected with their metabolism.

But we must strongly emphasize the point that knowledge gained from such a study alone is necessarily very incomplete. Suppose, for the sake of argument, that it is found that the concentration of some particular constituent, say phosphatide, in the blood is highly variable; and that it is desired to determine the factors on which this variability depends. The most cursory consideration will show that these factors are numerous and not easily controlled. Steps taken to alter any one of them may have unexpected effects on several of the others, and any of them may be altered during the course of an experiment by external or internal influences which it is impossible to keep under control. But in spite of these difficulties it has seemed to us worth while to attempt this line of investigation, and we think that the results already attained have at least a certain value as indications of the problems which ought in the future to be attacked.

Calcium of Blood.

The literature on the variations which occur in the concentration of calcium in the blood under normal conditions is not very large, and can be profitably considered in connection with our own results.

It is universally agreed, as far as we know, that the calcium of mammalian blood is contained in the plasma, and to a negligibly small extent, if at all, in the corpuscles. Our own experience falls in with this view; we think it justifiable to regard it as established. In our discussion we shall consider only the concentration of calcium in the plasma or serum, omitting any work which bears exclusively on the calcium of whole blood. We shall give our figures always in terms of elemental Ca; where the figures in the original articles were given as CaO, we shall calculate them back to Ca for the purpose of our discussion.

Halverson, Mohler, and Bergeim[46] have studied the calcium content of the serum in normal human beings and in patients suffering from various diseases. They worked also to some extent on plasma and found that

[46] Halverson, J. O., Mohler, H. K., and Bergeim, O., *J. Am. Med. Assn.*, 1917, lxviii, 1309; *J. Biol. Chem.*, 1917, xxxii, 171.

there was no appreciable difference in the calcium content of plasma and serum. In normal human beings they found that the calcium ranged between 9 and 11 mg. per 100 cc. of plasma; and, in their whole series of tuberculous patients, it ranged only from 8.4 to 11 mg. Their experience indicates that the calcium content of the serum is little affected by calcium given with the food. It was not in any case increased above normal figures by a high calcium diet.

Somewhat contradictory of this last result are some experiments on calcium feeding reported by Boggs,[47] who fed calcium lactate to dogs, and found as a result that the calcium of the blood increased from 6.3 mg. per 100 cc. to 8.6 mg., an increase of 36 per cent. It is very unlikely that feeding calcium lactate would produce any marked change in the volume of the corpuscles, and these results, therefore, seem clearly to indicate a change in the concentration of plasma calcium as the result of calcium feeding. Boggs determined the calcium in the blood and plasma of dogs kept for some time on a diet of bread and meat, and found that under these conditions it varied little. His figure for the normal calcium content of the plasma is 10 mg. per 100 cc.

Lamers[48] has made a more or less extended study of the concentration of calcium in the plasma of pregnant women and of women in labor as compared with that of non-pregnant women of child bearing age. Fourteen non-pregnant women had an average concentration of 10.8 mg. of calcium per 100 gm. of plasma; the figures ranged from 9.9 to 12.1 mg. For thirteen pregnant women the average was 11.6 mg.; the lowest figure was 10.6 mg., and the highest 12.8 mg. For eight women in labor the average was 12.8, the lowest figure 11.5 mg., and the highest 14.1 mg.

Determinations of the calcium content of horse serum have recently been made by Rona and Takahashi.[22] They find that under ordinary circumstances horse serum contains about 12 mg. of calcium per 100 gm.

We have determined the calcium content of plasma in pregnant and lactating mature cows and in heifers of various ages from birth onward. We have also carried out experiments to determine the effects of feeding a soluble calcium salt (calcium chloride) on the calcium concentration of the plasma. Our results for mature cows are given in Table II. They indicate that in cows the calcium content of the plasma is quite constant and not affected by either pregnancy or lactation.

The results of calcium determinations in the plasma of heifers at various ages are given under Experiments 11, 12, 13, 14, and 15. For the most part these results lend further support to the view that the plasma calcium of cattle is quite constant, and they

[48] Boggs, T. R., *Bull. Johns Hopkins Hosp.*, 1908, xix, 201.
[47] Lamers, A. J. M., *Z. Geburtsh. u. Gynäk.*, 1912, lxxi. 393.

make it appear that in cattle there is no rise in the concentration of this element as the result of pregnancy. The figures for the farrow heifers average higher than those for the pregnant cows. We do not wish to imply, however, that we think Lamers' results are purely a matter of chance or caused by experimental error. There are at least two important differences between his experiments and ours. One of these is that the subjects of his experiments were probably on an unrestricted diet, while the rations of cows are always restricted; the other, that his pregnant and nonpregnant women were probably much more nearly of the same average age than were our heifers and pregnant mature cows.

TABLE II.

Calcium in Plasma of Pregnant and Lactating Cows. Mg. per 100 Gm. of Plasma.

Pregnant, not lactating.			Pregnant and lactating.				Lactating, not pregnant.		
No. of cow.	Plasma calcium.	Days pregnant.	No. of cow.	Plasma calcium.	Days pregnant.	Days lactating.	No. of cow.	Plasma calcium.	Days lactating.
	mg.			*mg.*				*mg.*	
9	9.6	270	21	9.1	36	152	9	8.9	26
19	10.2	236	35	9.9	45	91	19	10.2	21
27	8.9*	234	54	9.7	176	254	106	9.8	220
48	9.7	251					107	10.2	40
Average.	9.6			9.6				9.8	

* This figure represents the average of the following four determinations. Check 1, 9.5; check 2, 8.9; check 3, 8.7; check 4, 8.7. All other figures for calcium in this table are based on duplicates which checked within 5 per cent.

Our experiments indicate that the plasma calcium tends to become lower with advancing age, at least up to 6 months. In three of them (Experiments 11, 12, and 13) we secured blood samples when the heifers were only 1 or 2 days old, and in two of these cases, the plasma calcium was decidedly higher than in any of the other samples that we have examined—13.4 and 13.7 mg. per 100 gm. of plasma respectively. The heifers which gave these high figures were afterward fed calcium chloride and sodium phosphate respectively; but, at the time the samples were taken, they had had nothing to eat except the small amount of colostrum which a new born heifer is likely to take from her dam.

Heifer 81, Experiment 12, was fed calcium chloride in addition to the usual rations throughout the period of the experiment (see protocols). Her plasma calcium was a little higher on the average than that of the other three heifers, but the difference is not great.

Under Experiment 7 are given the results of a short time calcium feeding experiment, more or less comparable to those of Boggs mentioned above. There was a very slight increase in the plasma calcium after the calcium feeding but the difference was within the limits of experimental error for our single determinations.

The results so far given would make it appear that in adult human beings and in cattle above the age of 2 months the plasma calcium is little if at all altered by pregnancy or lactation or by feeding rations rich in calcium. We are inclined to agree with the suggestion given by Halverson, Mohler, and Bergeim that, under ordinary conditions, the plasma calcium is kept nearly constant by means of the enormous calcium reserve stored in the bones.

But the high figures which we obtained in the case of the new born heifers call for an explanation; and we think that the key to the situation is given in an interesting article by Allers and Bondi,[49] who found that they could double the concentration of calcium in the blood of rabbits by feeding moderate doses of hydrochloric acid.

In a series of recent articles by Van Slyke and his associates[50] it has been shown that the best criterion for acidosis is the concentration of bicarbonate in the blood plasma. In acidotic conditions, the bicarbonate concentration of the plasma is uniformly depressed. Feeding acid, therefore, would tend to depress the bicarbonate concentration in the plasma, and that an inverse relation between the calcium and bicarbonate contents of the plasma is highly probable is shown by the work of Rona and Takahashi.[22] These authors studied various mixtures of calcium

[49] Allers, R. A., and Bondi, S., *Biochem. Z.*, 1907, vi, 366.

[50] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289. Van Slyke, D. D., *ibid.*, 347. Cullen, G. E., *ibid.*, 369. Fitz, R., and Van Slyke, D. D., *ibid.*, 389. Van Slyke, D. D., Stillman, E., and Cullen, G. E., *ibid.*, 401. Stillman, E., Van Slyke, D. D., Cullen, G. E., and Fitz, R., *ibid.*, 405.

and bicarbonate by physicochemical methods and found that the relation between the hydrogen ion concentration and the calcium and bicarbonate contents of such mixtures was expressed by the equation, $\frac{(Ca'')(HCO'_3)}{(H')} = a$ constant. The equation means, of course, that if the hydrogen ion concentration remains constant the amount of calcium held in solution will vary inversely with the bicarbonate.

The physiological results which are at present at hand accord very satisfactorily with the view that the calcium and bicarbonate concentrations of plasma tend to vary inversely. The most striking, of course, are those of Allers and Bondi referred to above. It may well be that the bicarbonate concentration of the blood in their experiments was reduced to half by the acid feeding.

Lamers' results showing an increased plasma calcium in pregnancy are to be placed side by side with the work of Hasselbalch and Gammeltoft,[51] who showed that in pregnancy in human beings there is a definite tendency for the hydrogen ion concentration of the blood at a fixed CO_2 tension to be increased. And his results on the high blood calcium of women in labor are to be considered in connection with the well known tendency of severe muscular effort to cause lactic acid to be thrown into the blood. Finally our own results, indicating a high calcium content in the plasma of very young animals, are to be taken in connection with Marriott's conclusion that the blood of infants has a higher "fixed acidity" than that of adults[52] and that in infants the alveolar CO_2 tension is low.[53] We hope at a later date to compare the Ca and HCO_3 content of the same samples of plasma taken under various conditions.

Phosphorus of Blood.

The phosphorus of the blood, unlike the calcium, is contained in greater concentration in the corpuscles than in the plasma. If

[51] Hasselbalch, K. A., and Gammeltoft, S. A., *Biochem. Z.*, 1915, lxviii, 206.

[52] Marriott, W. McK., *Arch. Int. Med.*, 1916, xvii, 847.

[53] Marriott, *J. Am. Med. Assn.*, 1916, lxvi, 1596.

one looks in Abderhalden's tables[54] for the phosphorus content of the blood in various domesticated mammals, one finds that the corpuscle content varies widely in different animals. But one gets the impression from Abderhalden's figures that the phosphorus content of the serum is rather constant.

Greenwald,[55] however, has recently reported a series of twelve determinations of total, lipoid, and "acid-soluble" phosphorus in the blood serum of several normal human beings. It appears from his figures that the phosphorus of the serum is quite variable. The total varies from 9.54 to 19.65 mg. per 100 cc. of serum; the lipoid phosphorus, from 6.64 to 12.95 mg., and the "acid-soluble," from 1.97 to 6.80 mg. Very large variations occur in the "acid-soluble" phosphorus of the serum of the same individual collected at different times.

It was clear from the beginning of our work on the phosphorus content of the blood in cattle that both the corpuscle and the plasma phosphorus were highly variable, not only from individual to individual, but also in the same individual examined at different times. We have endeavored to determine some of the factors on which this variability depends.

The subject, as will be seen, is rather complicated. We have to consider separately the phosphorus of the plasma and that of the corpuscles, and each of these fractions of the blood contains two or three well marked classes of phosphorus compounds. In our work we have laid the chief emphasis on the plasma phosphorus, but in each sample of blood, we have determined the volume of the corpuscles and the total phosphorus for the whole blood as a matter of routine; from these figures, in connection with the figure for the total phosphorus of the plasma, the total phosphorus contained in the corpuscles may be calculated. In some samples of blood we have determined the lipoid phosphorus of the whole blood as well as that of the plasma, and, from our figures for these samples, the lipoid phosphorus of the corpuscles can be calculated. In much of our later work, the lipoid and inorganic phosphorus have been directly determined in each sample of plasma.

[54] Abderhalden, *Z. physiol. Chem.*, 1898, xxv, 65.
[55] Greenwald, *J. Biol. Chem.*, 1915, xxi, 29.

We have studied particularly the changes which are produced in the phosphorus of the blood by increasing age, by changes in ration, and by pregnancy and lactation. It will be well to begin this part of our report by stating that all these changes produce effects, and that the problem is, therefore, one in which the influences of a number of factors, which may either reinforce or neutralize each other, must be disentangled.

Effects of Increasing Age on Blood Phosphorus.

Some idea of the changes which occur in the total phosphorus content of the corpuscles and in the lipoid and inorganic phosphorus content of the plasma as the accompaniment of increasing age may be gained by studying the figures given in Experiments 11 to 15.

For the first few days after birth the corpuscle phosphorus is very high—about twice as high as in older animals. This condition disappears rapidly; it is much diminished at the end of 2 months and hardly noticeable at the end of 4. The lipoid phosphorus in the plasma of new born calves is very low, and increases gradually up to the age of about a year. The inorganic phosphorus of the plasma is fairly high in the new born calves, but tends to increase for some time and reaches a maximum at about the age of 6 months. It is likely to fall off again at some time between this age and that of 18 months.

Effects of Change in Rations on Blood Phosphorus.

We have two experiments, Nos. 9 and 10, in which the effects of a change in rations on the phosphorus content of the blood are quite clearly shown. The subjects were two cows which were near the height of lactation. Blood samples were obtained after they had been on the comparatively high grain and hay rations ordinarily fed to the milking cows at Beltsville, Md., and again at 10 day intervals while the grain and hay rations were very much cut down—cut down, that is, to the amount usually fed to the dry cows.[56] In these experiments we followed the total phos-

[56] Most of the phosphorus in the rations fed to these cows was contained in the grain.

phorus in both blood and plasma, the volume per cent of the blood corpuscles, the lipoid and inorganic phosphorus and the nitrogen in the plasma, and we calculated the total phosphorus in the corpuscles. In addition we followed the milk yields.

As was to have been expected, the low feeding produced no consistent changes in the nitrogen content of the plasma. But the results in the cases of the other constituents studied are quite marked and consistent, and they seem to us significant.

In the case of Cow 39, Experiment 10, the rations were cut down to a lower point than in the other experiment and the results are more marked and consistent. We shall, therefore, consider her case first, and later discuss that of Cow 33 in connection with it.

In Cow 39 the total phosphorus in both blood and plasma, the lipoid and inorganic phosphorus in the plasma, the total phosphorus in the corpuscles, and the volume per cent of the corpuscles fell off during the period of low feeding and recovered when the cow was put back on higher rations. The milk yield also fell off quite markedly during the period of low feeding, and recovered subsequently, though not to the level which it had before the low feeding was begun. Fig. 1 gives a graphic representation of the changes in lipoid and inorganic phosphorus in the plasma which occurred in this experiment.

The changes in the lipoid phosphorus in the plasma are decidedly less marked than those in the inorganic; they do not occur until later, and the recovery begins before the period of low feeding is finished. During the subsequent period of high feeding, the lipoid phosphorus shoots up above the concentration which it had at the beginning of the experiment.

The results in the case of Cow 33 are more or less the same as those that have just been discussed, except that they are smaller and more irregular, and that there appears to be a marked discrepancy between the two experiments in regard to the changes which took place in the concentrations of lipoid and inorganic phosphorus in the plasma during the first 10 days of the low feeding. The figures would make it appear that the lipoid phosphorus decreased markedly in the case of Cow 33 during this period, while the inorganic phosphorus increased.

The figure for inorganic phosphorus for the plasma of this cow obtained September 27, 1916, (at the end of the preliminary period

of high feeding) is calculated by subtracting the lipoid from the total phosphorus of the same sample, and we are inclined to question the figure given for lipoid phosphorus. It is true that this figure is based upon two concordant determinations by the nephelometric method, but they were among the first that we made by this method; and, in the light of our later experience, we should not be surprised if, using the procedure that we then used, we got two concordant results, both of which were 10 or 20 per cent off. If the figures are correct, they indicate an unusual temporary variation in the lipoid and inorganic phosphorus of the

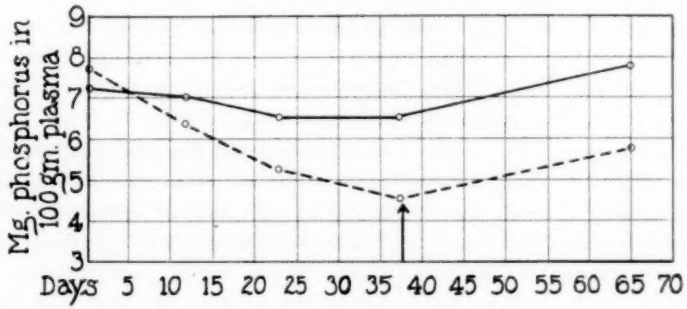

FIG. 1. Changes in the lipoid and inorganic phosphorus content of the plasma which are brought about by a reduction in the grain and hay rations of a milking cow. The solid line represents the lipoid phosphorus; the broken line, the inorganic phosphorus. The rations were reduced immediately after the first determinations of plasma phosphorus had been made, and were restored to the original level at the point marked by the arrow. See Experiment 10, Cow 39.

plasma. It is contrary to all our experience that feeding a reduced ration should be accompanied by an increase in the inorganic phosphate of the plasma.

In view of our present very scanty knowledge of the physicochemical nature of secretion and of the causal connections between rates of secretions and concentrations of the precursors of gland products in the blood, a detailed discussion of the results of these experiments would be unprofitable. But the results seem to us to suggest certain views. We shall consider, chiefly in this discussion, the case of Cow 39, on account of the unfortunate doubt concerning the preliminary figures for Cow 33.

The results suggest, in the first place, that the amount of milk secreted is independent of the concentration of lipoid phosphorus in the plasma, at least within the limits of concentration encountered in this experiment. In the early part of the experiment the milk yield drops off sharply; and the plasma phosphatide, hardly at all; while, during the final high feeding period, the phosphatide shoots above the concentration that it had at the beginning and the milk yield only partially recovers toward its original level. We do not think this at all inconsistent with the view that phosphatide is the precursor of milk fat and milk phosphorus. These are only two of many milk constituents, and it is probable that the total amount of milk secreted depends on quite other factors. During the period of low feeding, there is a more or less clearly marked inverse relation between the concentration of phosphatide in the plasma and the daily milk yield. We are inclined to explain this by supposing that the phosphatide was poured into the plasma at a nearly constant rate during this period, and that its concentration in the plasma therefore varied inversely with the rate at which it was excreted in the milk. The same sort of argument applies to the rise in plasma phosphatide above its original level during the subsequent period of high feeding. Here the high feeding caused the phosphatide to be poured into the plasma at approximately the same rate as in the preliminary period of high feeding, but, as the daily milk yield was decidedly less, the concentration rose even above its original level. This part of the discussion applies with equal force to both experiments.

The effects on the milk yield of decreasing and then increasing the rations are clearly marked in both experiments, and we cannot help being impressed with the parallelism exhibited between the daily milk yield and the concentration of inorganic phosphate in the plasma. The parallelism occurs throughout the experiment on Cow 39 and throughout that on No. 33 also, except for the doubtful preliminary figures which have been already discussed. We are not ready, however, to draw the conclusion that the concentration of inorganic phosphate in the plasma has an influence on the milk yield. The reduction in milk yield and in plasma phosphate may have been parallel effects of the reduction in the grain and hay rations, which had nothing directly to

do with one another. We are at present carrying out further experiments along this line.

The results given in Experiments 11, 12, 13, and 14 indicate that feeding a soluble inorganic phosphate tends to raise the concentration of the plasma phosphate in heifers. The subjects of these experiments were four heifers, which were all fed the same basic rations from birth onward. One, however, Heifer 119, was given daily doses of disodium phosphate in addition, and another, No. 81, daily doses of calcium chloride. The other two were fed nothing in addition to the basic rations. The figures for inorganic phosphate in the plasma of Heifer 119 are decidedly higher on the average than those for either of the controls or for the calcium heifer. It is not to be expected that the difference would be very marked in such experiments as these, for the control rations contained what is generally supposed to be a quite adequate amount of phosphorus.

It is an interesting fact that the plasma of the heifer fed calcium contained less inorganic phosphate on the average than that of any of the others. That feeding calcium chloride tends to reduce the inorganic phosphate in the plasma is shown again in the results of Experiment 7. It is very well known that the feeding of soluble calcium salts tends to reduce the amount of phosphorus excreted in the urine and to increase that excreted in the feces. It seems to us the most natural explanation for these results, as well as for ours on the blood, that calcium interferes with the absorption of phosphates by precipitating them as insoluble calcium phosphate within the gut.

Effects of Pregnancy on Blood Phosphorus.

We have as yet no data for the changes in the phosphorus content of the blood which occur in the beginning of pregnancy uncomplicated by lactation. But we have figures which indicate that toward the end of pregnancy the phosphorus content of the blood and plasma is likely to be reduced. In the case of Cows 19 and 39, Experiments 8 and 10, we secured blood samples which show this tendency. Both these cows were on a constant ration. The blood samples under discussion were secured January 30, and March 9, 1917, in the case of Cow 19, and May 28 and July

24, 1917, in the case of Cow 39. All four samples were obtained after lactation had ceased. In both cases the total and inorganic phosphorus of the plasma falls off as pregnancy proceeds (Experiments 8 and 10).

In four other cases we noted a drop in the phosphorus content of the blood or plasma toward the end of pregnancy, although the phosphorus-containing portions of the rations were increased after the first blood samples had been taken. One of these cases is given under Experiment 15; the blood samples in question are those obtained March 20 and May 2, 1918. Three other cases occurred in experiments which we have not thought it worth while to report in detail. The phosphorus in the blood and plasma fell off toward the end of pregnancy in spite of increases in the phosphorus-containing portions of the rations.

We have no contrary results, and we are inclined to regard it as established that there is a tendency for the phosphorus content of the blood and plasma to fall off toward the end of pregnancy—a tendency which is largely independent of the rations, and which shows itself most constantly in the inorganic phosphate of the plasma. The point may be of considerable practical importance. It would be unprofitable to discuss it at any length in the present scanty state of our knowledge, but it seems worth while to recall the fact that, toward the end of pregnancy, phosphorus is rapidly deposited in the bones of the growing embryo.[57] Whether this alone accounts for the reduction of phosphorus in the blood, or whether it is reinforced by a decreased ability to absorb phosphorus or by an increased tendency to excrete it in the urine and feces, the hint that cows may often require more of this element than they get under ordinary methods of feeding toward the end of pregnancy would seem to be equally plain.

Effects of Lactation on Blood Phosphorus.

In order to determine the changes in the phosphorus content of the blood which accompany the transition from pregnancy to lactation, we carried out three experiments in which cows were

[57] Forbes and Keith, A review of the literature of phosphorus compounds in animal metabolism, *Ohio Agric. Exp. Sta., Technical Bull. 5*, 1914, 110.

kept on constant or nearly constant rations through the latter part of one lactation period, the succeeding dry period, and the early part of the next lactation period; and in which the total phosphorus in the blood, and the lipoid and inorganic phosphorus in the plasma were followed. The results are given under Experiments 8, 9, and 10.

The changes which occur in the phosphorus of the plasma are quite striking in these experiments, and we shall discuss them first. A graphic representation of them is given in Figs. 2, 3,

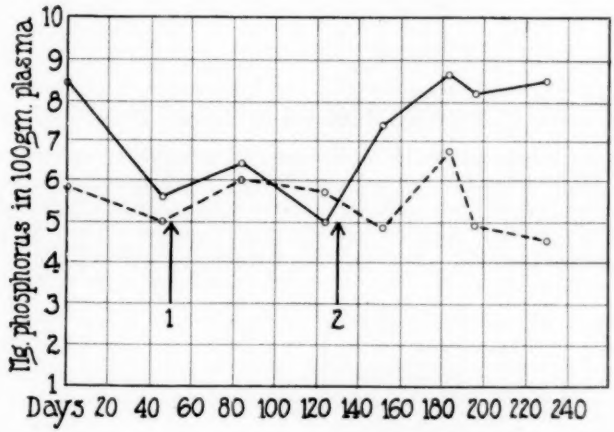

FIG. 2. Changes in the lipoid and inorganic phosphorus content of the plasma which occur as the accompaniment of pregnancy and lactation on a nearly constant diet. The solid line represents the lipoid phosphorus; the broken line, the inorganic phosphorus. At arrow 1 the cow became dry; at arrow 2 her calf was born. See Experiment 8, Cow 19.

and 4, both lipoid and inorganic phosphorus included. In all three cases the lipoid phosphorus falls off quite markedly at the end of the first lactation period, remains low during the dry period, and rises again in the second lactation period. The rise in the second lactation period does not occur immediately (Figs. 3 and 4) but is marked at the end of 28 days (Fig. 2). The changes in the inorganic phosphorus are rather irregular; in two cases (Figs. 2 and 3), it tends to fall off in the early period of lactation when the lipoid phosphorus is rising. It is to be noted that the absolute figures for lipoid phosphorus in these three cows in their

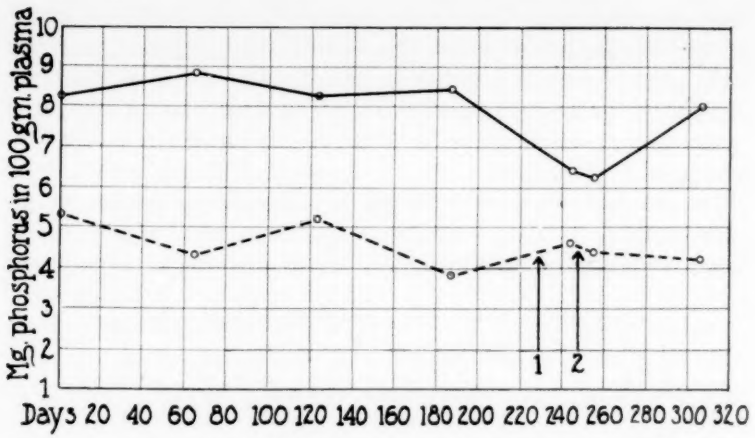

FIG. 3. Changes in the lipoid and inorganic phosphorus content of the plasma, which occur as the accompaniment of pregnancy and lactation on a nearly constant diet. The solid line represents the lipoid phosphorus; the broken line, the inorganic phosphorus. At arrow 1 the cow became dry; at arrow 2 her calf was born. See Experiment 9, Cow 33.

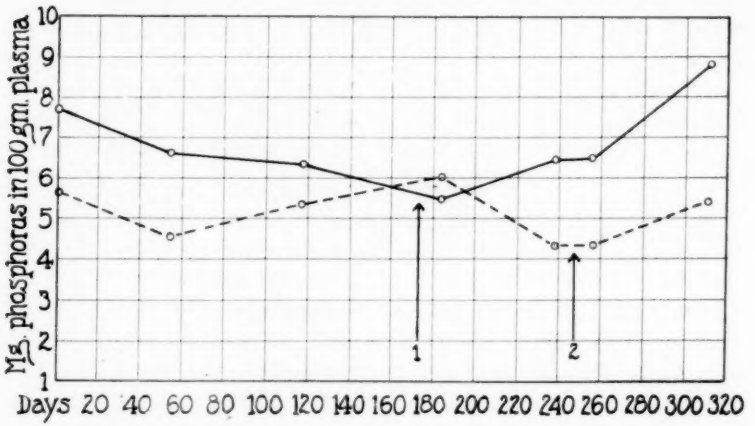

FIG. 4. Changes in the lipoid and inorganic phosphorus content of the plasma, which occur as the accompaniment of pregnancy and lactation on a nearly constant diet. The unbroken line represents the lipoid phosphorus; the broken line, the inorganic phosphorus. At arrow 1 the cow became dry; at arrow 2 her calf was born. See Experiment 10, Cow 39.

milking periods are considerably higher than any figures that we have ever obtained in non-milking animals. They run, for the most part, between 8 and 9 mg. per 100 gm. of plasma, and we have never encountered a lipoid phosphorus as high as 7 mg. per 100 gm. in any non-milking animal.

For the changes in the volume of the corpuscles, in the total phosphorus of the corpuscles, and in the nitrogen of the plasma which accompany the transition from pregnancy to lactation, the reader is referred to the figures given in the experiments. These changes do not seem to us sufficiently regular to call for any discussion.

We have a few results which bear on the lipoid phosphorus of the corpuscles and on the total fat of the plasma as influenced by lactation. It has not been convenient to include these results in the protocols of the experiments, but we think they are sufficiently interesting to warrant our giving a report of them here.

In the case of Cow 19, the lipoid phosphorus of the whole blood was determined on March 9, 1917, 3 days before she had her calf, and again on April 9 and June 22, 1917, after she had been milking 28 and 102 days respectively. From these figures the lipoid phosphorus of the corpuscles can be calculated; it was 0.0137 per cent on March 9, 0.0139 per cent on April 9, and 0.0152 per cent on June 22. The figures indicate, as far as they go, that the phosphatide content of the corpuscles tends to rise in lactation, somewhat as does that of the plasma, though to a less extent.

Total fat was determined in the plasma of Cow 19 on January 30, 1917, 41 days before she calved, and again on May 9, after she had been milking for 58 days. The determinations were made by the nephelometric method referred to in the description of our methods. It was found that on January 30 the total plasma fat was 543 mg. per 100 gm.; on May 9, 783 mg. per 100 gm. On these dates the lipoid phosphorus content of her plasma was 6.4 mg. and 8.7 mg. per 100 gm. respectively. The ratio 543: 6.4 is about 85; that between 783 and 8.7 is 90; in other words, the ratio between the fat and phosphatide contents of the plasma tends to remain relatively constant through quite wide variations in the concentrations of the two materials. We have other results which show the same thing, but we do not think it

necessary to give them in detail. Bloor[58] and others have previously reached the conclusion that the ratio between total fatty acids and "lecithin" in the corpuscles and in the whole blood tends to remain constant.

Our results on the changes in plasma phosphorus and fat, which accompany the transition from pregnancy to lactation, may be summed up by saying that in the 1st month of lactation there is a marked tendency for the fat and phosphatide to rise, that this rise occurs even in the face of a reduced ration, and that it is likely to be accompanied by a fall in the concentration of inorganic phosphate in the plasma. There can be no doubt that fat and phosphorus are taken out of the blood more rapidly by the active mammary gland than by the unborn calf at any stage of its embryonic existence; and the results given therefore indicate that some physiological change occurs near the beginning of lactation which causes fat and phosphatide to be thrown into the blood more rapidly than they are under ordinary circumstances and that this condition persists until lactation ceases. We are inclined to connect the changes which have been demonstrated with the well known tendency of good dairy cows to get thin during their lactation period and to suppose that the primary change is a mobilization of the body fat.

General Considerations in Regard to Phosphorus and Fat Metabolism.

From the literature, from the results which have been reported, and from others that are not yet ready to report in detail, we have formed some hypotheses in regard to phosphorus and fat metabolism. We think it worth while to give an outline of them, though they cannot yet be regarded as definitely established.

The recent work of Bloor[59] on fat metabolism is so well known that no extended discussion of it need be given here. His results indicate that fat received by the body from the alimentary tract is very soon largely or completely converted into phospha-

[58] Bloor, *J. Biol. Chem.*, 1916, xxiv, 456; 1916, xxv, 582.

[59] Bloor, *J. Biol. Chem.*, 1914, xix, 1; 1915, xxiii, 317; 1916, xxiv, 447; 1916, xxv, 577. Bloor, W. R., and MacPherson, D. J., *ibid.*, 1917, xxxi, 79.

tide by the red blood corpuscles. This result implies that at least some of the organs and tissues of the body can receive their supply of fat and phosphorus from the blood only in the form of phosphatide. We think that the evidence already given in this article shows that this is the case for the mammary gland and for muscles.

There is satisfactory evidence independent of Bloor's to show that the body can synthesize phosphatide from triglycerides and inorganic phosphate.[60] To us it seems probable that the body not only can, but always does supply itself with phosphatide in this manner. Our results taken altogether give us a strong impression that it is the inorganic phosphate of the plasma which is chiefly influenced by changes in ration, and the same conclusion has been reached by Greenwald.[14] Such studies as have been made on the blood, therefore, indicate that it is only as inorganic phosphate that phosphorus from the digestive tract reaches the general circulation. But other considerations make it seem improbable that phosphatide introduced *per os* would escape being broken down into its components by the intestinal ferments, or that so complicated an organic compound would be received unchanged into the blood.

The facts given on pages 10 to 17 indicate that phosphorized proteins are not carried from one part of the body to another at all by the plasma, and they point to the conclusion that these compounds are always manufactured within the cells in which they are found.

We are inclined to think, then, that phosphorus from the digestive tract reaches the general circulation only in the form of inorganic phosphate, that all organic phosphorus compounds are synthesized within the body cells, and that phosphorized proteins are not transported at all by the plasma from one fixed cell in the body to another.

We think also that many of the tissues and organs of the body, of which the mammary gland and the muscles are conspicuous examples, can receive their fat and phosphorus from the blood only in the form of phosphatide.

[60] McCollum, E. V., Halpin, J. G., and Drescher, A. H., *J. Biol. Chem.*, 1912–13, xiii, 219. Fingerling, G., *Biochem. Z.*, 1912, xxxviii, 448.

SUMMARY.

1. Normal blood plasma contains no phosphorized proteins, and probably no phosphorus compounds at all except phosphatides and inorganic phosphates. The phosphorus of these two classes of compounds certainly comprises more than 97 per cent of all that exists in normal plasma.

2. The precursor in plasma of milk fat and milk phosphorus is phosphatide—either lecithin or some related body.

3. The concentration of calcium in the plasma of cows is quite constant. Small variations can be induced by varying the amount supplied with the rations, but the chief controlling factor is probably the concentration of bicarbonate in the plasma. It is probable that the concentration of calcium tends to vary inversely with that of the bicarbonate.

4. The concentrations of phosphatide and of inorganic phosphate in the plasma are highly variable. Both can be made to vary by changing the amount of phosphorous supplied with the rations, though the variations induced in this manner show themselves most markedly in the inorganic phosphate. Both undergo variations as the accompaniment of increasing age and of the later stages of pregnancy. The phosphatide of the plasma shows a marked tendency to rise during the 1st month of lactation and to remain high until lactation has ceased. This phenomenon is largely independent of the diet, and is thought to be connected with the fact that near the beginning of lactation there is a tendency for the body fat to be released from its stores and thrown out into the blood.

In conclusion we wish to express our most sincere gratitude to Mr. T. E. Woodward, Superintendent of the Dairy Division Farm at Beltsville, Md. Our work has been carried out entirely on this farm, and has been made possible largely through Mr. Woodward's interest and cooperation.

PROTOCOLS.

In order to save space we have not published the protocols of our chemical analyses in the usual way. Our analytical methods are described above, and the quantities of material which we took

for each kind of analysis are there indicated in a general way. We feel that this gives a sufficiently accurate idea of the amount of material that we had to weigh or titrate in each case.

We made duplicate determinations in all except certain special cases, in which our results were checked in some other way. For instance, after we found that the lipoid phosphorus in a sample of plasma could be calculated within the limits of error of our determinations by subtracting the inorganic from the total, we frequently made only one determination of the lipoid phosphorus in samples in which the total and inorganic were determined independently.

We have not published our duplicate figures, however, except in certain special cases. The figures given in our tables and protocols represent, unless otherwise indicated, the average of duplicates which agreed within 5 per cent.[61] Where our duplicates did not agree so closely as this, we have given the details in footnotes appended to the tables and protocols. We have published all our duplicate figures in Experiments 1 to 7. In these experiments, the differences to be demonstrated were close to our limits of error, and we feel that it is necessary to say a word about our special mode of procedure for these cases.

It has been our experience that if two analyses are carried along side by side, starting with the same quantities of the same materials and using measured and equal quantities of reagents throughout, the results are likely to agree closely with one another, even though the method may be faulty, and the two results may together diverge widely from what is known to be the correct one. For this reason, in making up our checks, we have usually taken rather different quantities of material. But, in comparing our jugular and mammary samples, we have taken equal quantities of each kind of blood or plasma, our method being as follows: Equal samples of jugular and mammary plasma or blood are weighed out, and the two are carried along as nearly as possible exactly parallel. This set of analyses is checked by another set, consisting again of a sample of jugular plasma and one of mammary plasma, the two being equal to each other, but

[61] *I.e.*, the difference obtained by subtracting one figure from the other was 5 per cent or less of the larger.

both different in amount from the first set. Throughout the experiments, we have endeavored to carry through each set consisting of one jugular and one mammary sample at the same time and as nearly as possible under the same conditions; in the case of the check sets, no special care was taken to make them parallel to their checks, and the amounts of material were purposely chosen different. It seems to us that if, in such experiments, the jugular and mammary samples differ from each other in phosphorus content more than they do from their own checks, it is justifiable to assume that a difference in the phosphorus content of the jugular and mammary samples has been demonstrated.

Experiment 1. Comparison of Jugular and Mammary Plasma Obtained from Cow 19.—The animal had calved normally on March 12, 1917, and, at the time of the experiment (May 9), was giving 29 pounds of milk daily with a fat content of about 5 per cent. She was milked for the last time before the experiment at 6.00 p.m. on May 8. At 8.00 a.m., May 9, her hind legs were tied together to prevent kicking, and a trochar and cannula were inserted in her jugular vein. The cannula was left stoppered in the vein, and an attempt was made to insert another trochar and cannula in the milk vein. Before this could be done, however, the cow struggled and fell down in the stall; it was then found that blood would not run from the jugular cannula, and this was removed.

A trochar and cannula were inserted in the milk vein, and the cannula was left stoppered in the vein while the cow was milked. The milk did not run freely, but about 6 pounds were obtained; 500 cc. of blood were then collected from the cannula while the cow was still being milked; the blood ran very freely. Immediately after finishing the collection of the mammary blood, a trochar and cannula were inserted in the jugular vein and 500 cc. of blood were obtained from it, the milking being continued through the whole process. About a minute elapsed between the ending of the collection of the mammary blood and the beginning of that of the jugular blood. Both samples of blood were oxalated and centrifuged, and the two samples of plasma were analyzed for total, lipoid, and inorganic phosphorus, and for nitrogen.

Results in Mg. per 100 Gm. of Plasma.

Total phosphorus.		Lipoid phosphorus.	
Jugular.	Mammary.	Jugular.	Mammary.
I 15.62	16.36	8.79	8.79
II 15.70	16.32	8.70	8.70
Average. 15.66	16.34	8.74	8.74
Total phosphorus increased 0.68 mg. per 100 gm. in mammary plasma.		Lipoid phosphorus equal in jugular and mammary plasma.	

Inorganic phosphorus.		Sum of lipoid and inorganic phosphorus concentrations, as compared with the total.	
Jugular.	Mammary.	Jugular.	Mammary.
I 6.79	7.56	Lipoid..... 8.74	8.74
II 6.85	7.39	Inorganic. 6.82	7.47
Average. 6.82	7.47		
Inorganic phosphorus increased 0.65 mg. per 100 gm. in mammary plasma.		Sum.....15.56	16.21
		Total..... 15.66	16.34

Nitrogen.	
Jugular.	Mammary.
I 1.253	1.253
II 1.230	1.234
Average. 1.241	1.243

The blood corpuscles had the same volume, 32.5 volumes per cent in both jugular and mammary blood.

Experiment 2. Comparison of Jugular and Mammary Blood and Plasma Obtained from Cow 211.—The animal had calved normally on July 25, 1917, and, at the time of the experiment (October 16), was giving 23 pounds of milk daily with a fat content of 2.9 per cent. She was milked about 4 hours before the experiment, and, during the collection of blood, her udder was not touched. All precautions were taken to have her disturbed as little as possible before and during the collection of the mammary blood. She and the other cows of the herd were left in their stalls from the time of the morning milking until the blood was collected between 8.30 and 9.00 a.m. The men who helped

in taking the blood were quiet. No precautions were taken to keep her in place, except that one of the men placed his arm in front of her hind legs to prevent kicking. She did not resent this and she made no attempt to kick; almost no sign of pain was

Mg. per 100 Gm. of Plasma or Blood.

Plasma.

Total phosphorus.		Lipoid phosphorus.	
Jugular.	Mammary.	Jugular.	Mammary.
I 11.71	11.97	7.49	6.89
II 12.07	11.81		
Average. 11.89	11.89	Lipoid phosphorus decreased 0.60 mg. per 100 gm. in mammary plasma.	
Total phosphorus equal in jugular and mammary plasma.			

Inorganic phosphorus.		Sum of lipoid and inorganic phosphorus concentrations as compared with total.	
Jugular.	Mammary.	Jugular.	Mammary.
I 3.95	4.31	Lipoid..... 7.49	6.89
II 4.08	4.58	Inorganic. 4.01	4.44
Average. 4.01	4.44	Sum..... 11.50	11.33
Inorganic phosphorus increased 0.43 mg. per 100 gm. in mammary plasma.		Total..... 11.89	11.89

Blood.

Total phosphorus.		Lipoid phosphorus.	
Jugular.	Mammary.	Jugular.	Mammary.
I 17.68	17.60	10.04	9.86
II 18.09	18.16	10.36	9.48
			9.89
Average. 17.88	17.88	10.20	9.74
Total phosphorus equal in jugular and mammary blood.		Lipoid phosphorus decreased 0.46 mg. per 100 gm. in mammary blood.	

The jugular blood contained 32.5 volumes per cent of corpuscles; the mammary, 30.9 volumes per cent.

shown when a trochar and cannula were inserted in her milk vein at 8.39 a.m. The cannula was left stoppered in the vein until 8.42 a.m., when about 575 cc. of blood were collected. The cow showed no sign of disturbance throughout this whole procedure. After the mammary blood had been collected, the cow's nose was secured and about 525 cc. of blood were collected from her jugular vein between 8.46 and 8.52 a.m. She was somewhat disturbed by this part of the procedure.

Both samples of blood were oxalated, and the greater part of each was centrifuged. The two samples of plasma were analyzed for total, lipoid, and inorganic phosphorus; the two portions of whole blood, which were not centrifuged, were analyzed for total and lipoid phosphorus.

Experiment 3. Comparison of Jugular and Mammary Blood and Plasma Obtained from Cow 17.—The animal calved normally on October 11, 1917, and, at the time of the experiment (November 1), was giving about 20 pounds of milk daily, with a fat content of 5.4 per cent. The calf had been kept with her dam and allowed to take milk from her at will from the time of her birth until the day of the experiment.

About 4.00 a.m., November 1, 1917, the calf was separated from her dam and kept without food. The cow, in the meantime, was allowed to stand in her stall, and disturbed as little as possible. At 10.50 a.m., a trochar and cannula were inserted in her milk vein as in the case of No. 211, Experiment 2. She moved a little when this was done, showing slightly more sign of pain than did Cow 211, but did not struggle or attempt to kick. The cannula was left stoppered in the vein, and the calf was brought in; the cow showed evident interest in the presence of her calf and stood quietly during the subsequent procedure. The calf was allowed to suck first for 1½ minutes on the side opposite to where the cannula had been inserted in the vein, and then for 1½ minutes, on the same side. 550 cc. of blood were then drawn while the calf was still sucking at intervals. The calf was somewhat disturbed by the presence of the experimenters and did not feed continuously but was actually sucking for about a quarter of the time that the mammary sample was being collected.

After the collection of the mammary sample, the cow's nose was secured, and 550 cc. of jugular blood were collected. The cow

was somewhat disturbed by this procedure, but much less than was No. 211 in Experiment 2. She stood quietly during the collection of the blood and chewed her cud a part of the time. During this part of the procedure, the calf sucked eagerly. Less than 5 minutes elapsed between the end of the collection of the mammary blood and the beginning of that of the jugular. The blood was treated and analyzed as in the preceding experiment.

Mg. per 100 Gm. of Plasma or Blood.

Plasma.

Total phosphorus.			Lipoid phosphorus.	
	Jugular.	Mammary.	Jugular.	Mammary.
I	10.43	10.55	6.01	5.27
II	10.17	10.77		
III	9.98	10.87		
Average.	10.19	10.73		
Total phosphorus increased 0.54 mg. per 100 gm. in mammary plasma.			Lipoid phosphorus decreased 0.74 mg. per 100 gm. in mammary plasma.	

Inorganic phosphorus.			Sum of lipoid and inorganic phosphorus concentrations as compared with total.	
	Jugular.	Mammary.	Jugular.	Mammary.
I	4.08	5.13	Lipoid.... 6.01	5.27
II	4.12	5.25	Inorganic. 4.10	5.19
Average.	4.10	5.19	Sum..... 10.11	10.46
			Total..... 10.19	10.73
Inorganic phosphorus increased 1.09 mg. per 100 gm. in mammary plasma.				

Blood.

Total phosphorus.			Lipoid phosphorus.	
	Jugular.	Mammary.	Jugular.	Mammary.
I	16.96	16.76	8.61	8.02
II	16.93	16.32		
Average.	16.94	16.54		
Total phosphorus decreased 0.40 mg. per 100 gm. in mammary blood.			Lipoid phosphorus decreased 0.59 mg. per 100 gm. in mammary blood.	

The jugular blood contained 31.4 volumes per cent of corpuscles; the mammary, 28.2 volumes per cent.

Experiment 4. Comparison of Jugular and Mammary Plasma and Blood Obtained from Cow 106.—The animal had calved on January 23, 1917, and had gone dry on December 30, 1917. She was due to calve again on February 17, 1918, but aborted on

Mg. per 100 Gm. of Plasma or Blood.

Plasma.

Total phosphorus.

	Jugular.	Mammary.
I	9.41	10.27
II	9.69	10.31
III		10.37
Average.	9.55	10.32

Total phosphorus increased 0.77 mg. per 100 gm. in mammary plasma.

Lipoid phosphorus.

Jugular.	Mammary.
4.91	4.89

Lipoid phosphorus decreased 0.02 mg. per 100 gm. in mammary plasma.

Inorganic phosphorus.

	Jugular.	Mammary.
I	4.57	5.20
II	4.53	5.07
Average.	4.55	5.13

Inorganic phosphorus increased 0.58 mg. per 100 gm. in mammary plasma.

Sum of lipoid and inorganic phosphorus concentrations as compared with total.

Jugular.		Mammary.
Lipoid....	4.91	4.89
Inorganic.	4.55	5.13
Sum.....	9.46	10.02
Total.....	9.55	10.32

Blood.

Total phosphorus.

	Jugular.	Mammary.
I	14.94	15.16
II	14.57	14.86
Average.	14.76	15.01

Total phosphorus increased 0.25 mg. per 100 gm. in mammary blood.

Lipoid phosphorus.

Jugular.	Mammary.
7.92	7.77

Lipoid phosphorus decreased 0.15 mg. per 100 gm. in mammary blood.

The jugular blood contained 31.4 volumes per cent of corpuscles; the mammary, 28.8 volumes per cent.

January 13, 1918, 9 days after the experiment, which was carried out January 4. Blood was obtained from her mammary and jugular veins essentially as in the case of No. 211, Experiment 2, the effort being made again to disturb her as little as possible. It was not so easy, however, to insert the trochar and cannula in her milk vein as it had been in the case of the milking cows, and she made two or three attempts to kick while this was being done. With this exception, the process of collecting blood ran about the same as in the case of Cow 211. It should be mentioned, however, that the blood ran decidedly less freely from the mammary cannula than in the case of the milking cows. 4 days after the experiment, it was found that milk could still easily be obtained from her udder. The blood was treated and analyzed as in the two preceding experiments.

Experiment 5. Comparison of Jugular and Mammary Plasma and Blood Obtained from Cow 115.—This animal was born November 7, 1914, and was, therefore, somewhat more than 3 years old on January 29, 1918, when the experiment was carried out. She had been bred at frequent intervals since August, 1916, but had never had a calf, and continued to come in heat frequently up to shortly before the time of the experiment. She was, therefore, probably not pregnant when the experiment was carried out. It was found possible, however, to get a few drops of milk from her udder on the day of the experiment. Her milk vein was much smaller than those of the milking cows or than those of the cows which were dry after a preceding period of lactation. Blood was obtained from her mammary and jugular veins essentially as in the case of Cows 211 and 106, Experiments 2 and 4, every effort being made to disturb her as little as possible by the experimental procedure. It was decidedly more difficult, however, to insert the mammary trochar and cannula than in any of the preceding experiments, and the cow made a number of vigorous attempts to kick while this was being done. The blood flowed less freely from the mammary cannula than in any of the preceding experiments. The jugular blood, however, was collected without any more difficulty than in the other experiments. The blood was treated and analyzed as in the two preceding experiments, except that the lipoid phosphorus was not determined in either the plasma or blood.

Mg. per 100 Gm. of Plasma or Blood.

Plasma.

	Total phosphorus.		Inorganic phosphorus.	
	Jugular.	Mammary.	Jugular.	Mammary.
I	10.74	11.61	5.47	6.18
II	11.07	11.37	5.39	6 07
Average.	10.90	11.49	5.43	6.12
	Total phosphorus increased 0.59 mg. per 100 gm. in mammary plasma.		Inorganic phosphorus increased 0.69 mg. per 100 gm. in mammary plasma.	

Blood.

	Total phosphorus.	
	Jugular.	Mammary.
I	16.94	17.25
II	17.65	17.18
Average.....	17.29	17.21

Total phosphorus decreased 0.08 mg. per 100 gm. in mammary blood.

The jugular blood contained 37.8 volumes per cent of corpuscles; the mammary, 33.5 volumes per cent.

Experiment 6. Comparison of Plasma and Blood Obtained from Cow 9 before and after Disturbance.—The animal had her last calf on March 12, 1916, had gone dry September 10, 1917, and was probably not pregnant at time of the experiment, February 14, 1918. A trochar and cannula were inserted in her left jugular vein in the usual manner, and the cannula was left stoppered in the vein for half an hour. She was allowed to stand in her stall during this period, secured only by a halter, and was kept under observation. It was noticed that she stood quietly and gave no sign that she noticed the cannula in her neck. At the end of the half hour the cannula was unstoppered and 550 cc. of blood were drawn from it; this procedure caused less disturbance than did the insertion of the mammary trochar and cannula in the cases of Cows 211 and 17, Experiments 2 and 3. The cow's nose was then secured, another trochar and cannula were inserted in the right jugular vein, and 550 cc. of blood were obtained from it.

The disturbance caused by this part of the procedure was about the same as that caused by the corresponding procedure in Experiment 2 and rather more than in the case of Experiment 3. About 7 minutes elapsed between the collections of the two samples of blood. The blood was treated and analyzed as in Experiments 2, 3, and 4.

Mg. per 100 Gm. of Plasma or Blood.

Plasma.

Total phosphorus.		Lipoid phosphorus.	
First jugular.	Second jugular.	First jugular.	Second jugular.
I 9.92	9.90	4.71	4.66
II 10.29	10.18	4.84	4.84
Average. 10.10	10.04	4.77	4.75
0.06 mg. per 100 gm. difference.		0.02 mg. per 100 gm. difference.	

Inorganic phosphorus.		Sum of lipoid and inorganic phosphorus concentrations as compared with total.	
First jugular.	Second jugular.	First jugular.	Second jugular.
I 5.62	5.35	Lipoid.... 4.77	4.75
II 5.55	5.39	Inorganic. 5.43	5.31
III 5.11	5.20		
Average. 5.43	5.31	Sum..... 10.20	10.06
0.12 mg. per 100 gm. difference.		Total..... 10.10	10.04

Blood.

Total phosphorus.		Lipoid phosphorus.	
First jugular.	Second jugular.	First jugular.	Second jugular.
I 15.67	15.79		9.13
II 16.16	16.22	8.78*	8.92
Average. 15.91	16.00		9.02
0.09 mg. per 100 gm. difference.		0.24 mg. per 100 gm. difference.	

* Only one determination made.

The first jugular blood contained 29.3 volumes per cent of corpuscles; the second jugular, 29.8 volumes per cent.

Experiment 7. Effects of Feeding Calcium Chloride on the Calcium and Phosphorus Content of the Blood Plasma.—On January 9, 1917, a blood sample was obtained from Animal 17, a grade steer, about 5 months old and weighing about 250 pounds. Its daily ration for some time previously had been 14 pounds of skimmed milk, 3 pounds of grain, consisting of 375 parts cornmeal, 200 parts wheat bran, and 100 parts linseed oil meal, 1 pound of alfalfa hay, and 8 pounds of corn silage. It was kept on this ration throughout the experiment, except for the addition of calcium chloride. From January 9 to 11, 1.8 gm. of calcium, as calcium chloride, were fed daily with the milk; and from January 11 to 15, 4.7 gm. The feeding of the calcium chloride produced no perceptible bad effects. On January 15, a second blood sample was obtained.

The two samples of blood were citrated and centrifuged. The whole blood was analyzed for total phosphorus; the plasma, for total and inorganic phosphorus, and for calcium.

Mg. per 100 Gm. of Blood or Plamsa.

	Total phosphorus in whole blood.		Total phosphorus in plasma.	
	Before calcium feeding.	After calcium feeding.	Before calcium feeding.	After calcium feeding.
I	19.65	18.38	10.80	9.56
II	19.21	18.66	10.64	10.42
Average.	19.43	18.52	10.72	9.99
	Total phosphorus decreased 0.91 mg. per 100 gm. after calcium feeding.		Total phosphorus decreased 0.73 mg. per 100 gm. after calcium feeding.	

	Inorganic phosphorus in plasma.		Calcium in plasma.	
	Before calcium feeding.	After calcium feeding.	Before calcium feeding.	After calcium feeding.
I	7.44	7.14	11.24	11.48
II	7.34	6.82	10.77	11.19
Average.	7.39	6.98	11.60	11.33
	Inorganic phosphorus decreased 0.41 mg. per 100 gm. after calcium feeding.		Calcium increased 0.33 mg. per 100 gm. after calcium feeding.	

Experiment 8. Cow 19.—This experiment shows the changes which occur in the phosphorus content of the blood and plasma as the accompaniment of pregnancy and lactation. The subject was a grade Jersey about 8 years old, and was kept on a nearly

Experiment 8. Cow 19.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Plasma.				Remarks.
				Phosphorus.				
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).	Nitrogen (gm. per 100 cc.).	
1916		*vol. per cent*						
Nov. 7	22.2*	39.3	33.8	13.9	8.5	5.9	1.35	Milking 229 days; 5 lbs. milk daily; pregnant, 154 days.
Dec. 22	18.8	40.8	28.8	11.1	5.7†	5.0	1.32	Milking 274 days; 2 lbs. milk daily; pregnant, 199 days.
1917								
Jan. 30	19.3‡	44.2	27.0	12.4	6.4†	6.0	1.32	Dry since Dec. 26, 1916; pregnant 238 days.
Mar. 9	19.5	42.1	29.9	11.0	5.0†	5.6	1.15	Pregnant 276 days.
Apr. 9	18.8	38.9	28.5	12.0	7.3†	4.9	1.25	Milking 28 days; 34.1 lbs. milk daily.
May 9	Not determined.	32.5	—	15.7	8.7	6.8	1.27	Milking 58 days; 29 lbs. milk daily.
" 22	" "	32.5	—	13.3	8.1	4.9§	1.24	Milking 71 days; 28 lbs. milk daily.
June 22	20.7	37.8	30.7	14.0	8.7	4.6‖	1.32	Milking 102 days; 25 lbs. milk daily.

* Check 1, 21.4; check 2, 23.0; check 3, 22.3; all three used in calculating average.

† One determination only.

‡ Check 1, 18.8; check 2, 19.8.

§ Check 1, 4.4; check 2, 4.8; check 3, 5.4; all three used in calculating average.

‖ Check 1, 4.3; check 2, 4.9; check 3, 4.5; all three used in calculating average.

constant ration from September 26, 1916, until July 9, 1917. From the former date until she calved on March 12, 1917, her daily ration was 10.8 pounds of grain (2 parts corn-meal, 1 part wheat bran, 1 part cottonseed meal), 7 pounds of alfalfa hay, 22 pounds of corn silage. For a few days immediately after she calved her ration was cut down, as is customary on most dairy farms; it was then resumed as above and continued until April 21, when her hay was reduced to 4 pounds daily, as she would not eat the 7 pounds which had previously been supplied. The hay was raised to 6 pounds on May 7, and this ration (10.8 pounds of grain as above, 6 pounds of alfalfa hay, 22 pounds of corn silage) was continued until July 9, 1917.

Experiment 9. Cow 33.—The latter part of this experiment is in general a repetition of Experiment 8, but with a lower ration. From April until November, 1917, the subject was kept on a nearly constant ration. She was fed daily 7 pounds of grain, consisting of 2 parts corn-meal, 2 parts wheat bran, and 1 part cottonseed meal, 6 pounds of alfalfa hay, and 25 pounds of corn silage. The small temporary changes which were made in this ration once or twice, on account of temporary loss of appetite or for other reasons, occurred at such times that they could have had no influence on the changes in the phosphorus content of the blood plasma which took place in connection with lactation and calving.

The first part of the experiment shows the changes in the phosphorus content of the blood and plasma which occurred as the accompaniment of a reduction in the rations. For 16 days before the first blood sample was obtained on September 27, 1916, her daily ration had been 7 pounds of grain (as above), 6 pounds of alfalfa hay, and 20 pounds of corn silage. From September 28 until November 2, inclusive, it was changed to 4 pounds of grain (as above), 4 pounds of alfalfa hay, and 25 pounds of corn silage. After November 2, it was restored to that fed before September 28.

Experiment 9. Cow 33.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Plasma.				Remarks
				Phosphorus.			Nitrogen (gm. per 100 cc.).	
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).		
1916		*vol. per cent*						
Sept. 27	20.3	34.6	32.5	13.3	8.6*	4.7†	1.21	Milking 42 days; 17.6 lbs. milk daily; high rations.
Oct. 9	19.7	33.9	31.6	13.1	7.5‡	5.6	1.30	Milking 54 days; 15.4 lbs. milk daily; low rations since Sept. 28.
" 19	20.4	33.5	34.6	12.7	7.1§	5.4	1.32	Milking 64 days; 15.7 lbs. milk daily; still on low rations.
Nov. 2	19.3	34.6	30.0	13.2	7.7‖	4.6	1.29	Milking 78 days; 15 lbs. milk daily; still on low rations.
Dec. 4	21.3	40.8	30.5	14.2¶	8.3§	5.4**	1.29	Milking 100 days; 16 lbs. milk daily; high rations since Nov. 3.
1917								
Feb. 5	20.3	41.5	29.0	13.4	8.9§	4.3	1.33	Milking 163 days; 14.5 lbs. milk daily.
Apr. 5	20.2	34.6	32.3	13.3	8.2‡‡	5.1	1.25	Milking 222 days; 12.2 lbs. milk daily.
June 8	19.5	33.6	32.3	12.5	8.6‡‡	3.9	1.24	Milking 286 days; 12.9 lbs. milk daily.
Aug. 3	19.1††	32.6	34.9	10.9	6.3‡‡	4.6	Not determined.	Dry since July 20; pregnant 284 days.

Experiment 9. Cow 33—Concluded.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Plasma.				Remarks.
				Phosphorus.			Nitrogen (gm. per 100 cc.).	
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).		
1917		*vol. per cent*						
Aug. 14	18.4	33.9	30.5	10.5§§	6.2‡‡	4.3	Not determined.	Milking 8 days; 14 lbs. milk daily.
Oct. 6	19.6	33.5	32.7	12.5***	8.0§	4.2	" "	Milking 61 days; 15 lbs. milk daily.

* Check 1, 9.6; check 2, 8.6; check 3, 8.6; check 4, 8.6; checks 2, 3, and 4 used in calculating average.

† Calculated by subtracting lipoid phosphorus from total.

‡ Figure based on one determination only; results of two other determinations were discarded, as they were obviously incorrect.

§ One determination only.

‖ Check 1, 7.5; check 2, 8.0.

¶ Check 1, 14.4; check 2, 13.2; check 3, 14.0; checks 1 and 3 used in calculating average.

** Check 1, 5.6; check 2, 5.2; check 3, 5.4; all three used in calculating average.

†† Check 1, 19.6; check 2, 18.5.

‡‡ Calculated by subtracting inorganic phosphorus from total.

§§ Check 1, 11.2; check 2, 10.4; check 3, 10.8; check 4, 10.5; checks 2, 3, and 4 used in calculating average.

*** Check 1, 13.3; check 2, 12.2; check 3, 12.6; check 4, 12.4; checks 3 and 4 used in calculating average.

Experiment 10. Cow 39.—This experiment is, in general, a repetition of Experiment 9. Here, as in Experiment 9, the first part shows the effect of reducing the rations on the phosphorus content of the blood and plasma and on the milk yield, while the second part shows the changes in the phosphorus content of the blood and plasma which occur as the accompaniment of pregnancy and lactation on a nearly constant ration.

From September 1 to 25, 1916, this cow was fed daily 6 pounds of grain (same as that fed to No. 33), 6 pounds of alfalfa hay, 15 pounds of corn silage. From September 26 until November 2, she was fed 3 pounds of grain (as above), 3 pounds of alfalfa hay,

Experiment 10. Cow 39.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Plasma.				Remarks.
				Phosphorus.			Nitrogen (gm. per 100 cc.).	
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).		
1916		*vol. per cent*						
Sept. 23	19.8	30.4	30.7	14.8	7.1*	7.7†	1.13	Milking 117 days; 14.2 lbs. milk daily; high rations.
Oct. 5	18.2	29.1	29.8	13.2	7.0*	6.2†	1.15	Milking 129 days; 10.6 lbs. milk daily; low rations since Sept. 25.
" 16	17.1*	29.3	28.6	12.1	6.4*	5.1	1.13	Milking 140 days; 11.2 lbs. milk daily; still on low rations.
" 30	15.8	29.2	26.4	11.2	6.5	4.6‡	1.14	Milking 154 days; 9.5 lbs. milk daily; still on low rations.
Nov. 27	19.0	34.6	28.8	13.4	7.7	5.7	1.16	Milking 182 days; 12.7 lbs. milk daily; high rations since Nov. 3.
1917								
Jan. 23	17.1	33.9	26.2	12.1	6.8*	4.6§	1.16	Milking 239 days; 10.3 lbs. milk daily.
Mar. 26	16.9	38.9	24.6	11.5	6.4	5.3*	1.15	Milking 301 days; 5.8 lbs. milk daily.
May 28	17.3	38.9	25.4	11.6	5.6‖	6.0	1.17	Dry since May 18; pregnant 230 days.
July 24	16.3	32.5	27.1	10.7	6.5‖	4.2	1.13	Pregnant 287 days.
Aug. 9	16.0	30.4	27.4	10.7	6.5‖	4.2	Not determined.	Milking 12 days; 20.2 lbs. milk daily.
Oct. 2	19.1	31.4	29.3	14.2	8.9‖	5.3	" "	Milking 66 days; 17.0 lbs. milk daily

* One determination only.

† Calculated by subtracting lipoid phosphorus from total.

‡ Check 1, 4.6; check 2, 3.9; check 3, 4.5; checks 1 and 3 used in calculating average.

§ Check 1, 4.8; check 2, 4.5.

‖ Calculated by subtracting inorganic phosphorus from total.

and 25 pounds of corn silage. After November 2 the ration was restored to that fed before September 25, and this ration was kept up (except for a reduction in the hay between April 21 and 30, 1917, on account of temporary loss of appetite) until she calved on July 28, 1917. It was cut down as usual for a few days after calving, then made 6 pounds of grain (as above), 6 pounds of alfalfa hay, and 30 pounds of corn silage. On September 10 the grain was increased to 8 pounds, and the silage was reduced to 25; this ration was continued until after October 2, 1917.

Experiments 11, 12, 13, and 14. Heifers 79, 81, 119, and 120.— These experiments show, in the first place, the changes which occur in the concentrations of various constituents of the blood and plasma as the result of advancing age. In the second place, they throw some light on the changes which can be produced in the concentrations of calcium and inorganic phosphate in the plasma by feeding either calcium chloride or sodium phosphate.

The four heifers, which served as subjects, were fed in general, as nearly as possible alike. They received rations like those recommended in the standard books on the subject, the object being to keep them growing and in good condition. This object was attained in all four cases.

The following is a rough description of the manner of feeding. 10 pounds of whole milk were fed daily up to the 10th day. Skimmed milk was then gradually substituted, and the calves got 12 pounds of this daily up to the 30th day. From the 30th to the 180th day the daily milk ration was 14 pounds; it was then rapidly reduced, and no more milk was fed after the 200th day. Small amounts of alfalfa hay were offered (and usually not eaten at first) from the 7th day on. From the end of the 3rd week small amounts of grain (375 parts corn and cob meal, 200 parts wheat bran, 100 parts linseed oil meal) and corn silage were also offered. When 60 days old the heifers were usually eating about a pound of grain, half a pound of hay, and 3 pounds of silage daily besides the milk. The grain, hay, and silage were gradually increased until at 300 days the calves were eating 3 pounds of grain, 3 pounds of alfalfa hay, and about 25 pounds of corn silage daily. The grain and hay were not increased beyond this point, but the silage was increased in some cases to as much as 32 pounds. Records were kept of the amounts of the various feeds eaten in each case. All the calves took practically all the milk and grain that was offered to them, so that the rations were nearly

similar in all four cases as far as these constituents were concerned. They also took all the hay after the first 4 or 5 weeks. There was some variation in the amount of silage eaten, but this was so small that the experiments may be regarded as comparable as far as the basic rations were concerned.

Heifers 79 and 120 were fed on the rations which have just been described, without additions. Heifer 81 received daily doses of calcium chloride in addition; and Heifer 119, daily doses of disodium phosphate. From February 20 to July 25, 1917, Heifer 81 received 2.6 gm. of Ca daily as calcium chloride; and from July 25 on, 5.7 gm. of Ca. From January 11 to March 19, 1917, Heifer 119 received 4.5 gm. of P daily as Na_2HPO_4, and from March 19 on, 6.9 gm. daily.

Heifer 119 showed no bad effects whatever as the result of the phosphate feeding, and Heifer 81 also showed no bad effects from the calcium feeding.

Experiment 11. Heifer 79, Fed Basic Ration without Addition.

Date.	Plasma.							Remarks.
	Blood, total P (mg. per 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Phosphorus.			Nitrogen (gm. per 100 cc.).	Calcium (mg. per 100 gm.).	
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).			
1916		*vol. per cent*							
Dec. 12	31.5	49.5	52.0	8.5	2.0*	6.3	0.959	11.2*	1 day old; born Dec. 11.
1917									
Feb. 17	20.0†	43.1	32.3	9.5	3.1*	6.4*	1.164	10.1	68 days old.
Apr. 16	22.0	42.0	32.8	13.2‡	4.2§	9.0	1.070	10.0	126 " "
July 19	20.9	34.6	33.6	13.6	6.9§	6.7	1.091	9.4	220 " "
Nov. 26	22.0	39.9	32.2	14.5	6.2§	8.3	Not determined.	10.1‖	350 " "
1918									
Apr. 29	17.9	37.8	27.6	11.4	5.8§	5.6	" "	9.4	1 yr., 139 days old.

* One determination only.

† Check 1, 20.8; check 2, 19.2.

‡ Check 1, 12.1; check 2, 13.2; check 3, 13.3; checks 2 and 3 used in calculating average.

§ Calculated by subtracting inorganic from total.

‖ Check 1, 10.5; check 2, 10.1; check 3, 10.0; check 4, 9.8; all four used in calculating average.

Experiment 12. Heifer 81, Fed Basic Ration plus Calcium Chloride.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Plasma.					Remarks.
				Phosphorus.			Nitrogen (gm. per 100 cc.).	Calcium (mg. per 100 gm.).	
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).			
1917		*vol. per cent*							
Feb. 20	29.5*	34.6	63.8	9.6†	2.6	6.0‡	1.148§	13.4	1 day old; born Feb. 19.
Apr. 20	17.5	31.4	39.5	9.4	2.2‖	7.2	1.038	10.4	60 days old.
June 19	18.3	34.1	29.7	11.9	4.9‖	7.0¶	0.918	10.6	120 " "
Sept. 22	19.0	38.9	28.9	12.0	4.1‖	7.9	Not determined.	10.5	215 " "
1918									
Jan. 24	17.9	37.8	28.1	11.1	4.4‖	6.7	" "	9.7	339 " "

* Figure based on one determination only; the result of a second determination was discarded as it was obviously incorrect.

† First two determinations discarded as they did not agree with one another; the figure given is based on another pair which was 9.74 and 9.46 respectively.

‡ Check 1, 6.3; check 2, 5.8.

§ Check 1, 1.112; check 2, 1.184.

‖ Calculated by subtracting inorganic from total.

¶ Check 1, 7.4; check 2, 6.7

Experiment 13. Heifer 119, Fed Basic Ration plus Disodium Phosphate.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Plasma.					Remarks.
				Phosphorus.			Nitrogen (gm. per 100 cc.).	Calcium (mg. per 100 gm.).	
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).			
1918		*vol. per cent*							
Jan. 11	28.1	36.7	56.4	10.0*	3.5†	6.3‡	1.146§	13.7	2 days old, born Jan. 9.
Mar. 12	20.8‖	36.7	34.6	12.0¶	3.3†	8.8†	0.975	10.8	62 days old.
May 14	21.2	41.5	33.2	11.7	3.2**	8.5	1.018	10.5	125 " "
Aug. 23	20.0	33.0	33.2	13.0	5.0**	8.0	Not determined.	10.1	226 " "
Dec. 3	21.6	41.5	31.3	13.9	6.5**	7.4	" "	10.8	328 " "

* Check 1, 11.1; check 2, 10.1; check 3, 9.9; checks 2 and 3 used in calculating average.

† One determination only.

‡ Check 1, 6.9; check 2, 5.9; check 3, 6.5; check 4, 6.2; checks 3 and 4 used in calculating average.

§ Check 1, 1.190; check 2, 1.102.

‖ Check 1, 21.6; check 2, 20.0.

¶ Check 1, 12.4; check 2, 12.1; check 3, 11.5; all three used in calculating average.

** Calculated by subtracting inorganic from total.

Experiment 14. Heifer 120, Fed Basic Ration without Addition.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Plasma.					Remarks.
				Phosphorus.			Nitrogen (gm. per 100 cc.).	Calcium (mg. per 100 gm.).	
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).			
1917		*vol. per cent*							
Apr. 24	17.7	33.0	35.1	8.4*	2.2†	6.2	1.052	9.7	62 days old, born Feb. 21.
June 29	19.7	38.9	30.6	12.0	5.4†	6.6	1.059	10.1	128 days old.
Sept. 27	22.0‡	34.6	33.4	15.5	6.3†	9.2	Not determined.	9.1	218 " "
1918									
Mar. 4	18.4	35.1	29.1	12.1	4.9†	7.2	" "	9.8	1 yr., 11 days old.

* Check 1, 8.6; check 2, 7.6; check 3, 8.2; checks 1 and 3 used in calculating average.

† Calculated by subtracting inorganic from total.

‡ Check 1, 22.8; check 2, 21.1.

Experiment 15. Heifer 214.—This experiment throws some further light on the changes in the calcium and phosphorus content of the blood and plasma which occur as the accompaniment of increasing age, of pregnancy, and of changes in ration.

The subject was a thoroughbred Holstein, born March 18, 1916. Up to August 4, 1917, no particular effort was made to control her rations, and she was fed about as the calves are usually fed at Beltsville, but with considerably more skimmed milk than was given to Heifers 79, 81, 119, and 120. From August 4 to September 10, 1917, she was fed daily 3 pounds of grain, consisting of $2\frac{1}{2}$ parts corn and cob meal, 2 parts wheat bran, 1 part cottonseed meal; 4 pounds of alfalfa hay; 15 pounds of corn silage. She became pregnant on August 23. On September 10, her silage was raised to 25 pounds.

On December 18, an experiment was started, one of the objects of which was to gain further light on the possibility of raising the concentration of inorganic phosphate in the blood plasma by feeding sodium phosphate. From this date until January 18, 1918, the heifer was fed 3 pounds of grain (as above), 4 pounds

Experiment 15. Heifer 214.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Plasma.					Remarks.
				Phosphorus.			Nitrogen (gm. per 100 cc.).	Calcium (mg. per 100 gm.).	
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.).			
1916		*vol. per cent*							
May 11	18.0	28.2	38.9	9.2	Not determined.	Not determined.	1.02	11.4	54 days old; born Mar. 18.
July 12	21.7	43.7	32.1	12.6	" "	" "	1.00	10.9	116 days old.
Aug. 21	21.4	44.9	30.2	13.2	" "	" "	0.98	10.6	156 " "
Nov. 20	19.6	30.5	34.6	12.7	4.7*	7.8	0.97	10.3	247 " "
1917									
Jan. 18	20.5	37.3	31.8	13.2	5.4†	6.1	1.03	10.7	306 " "
Mar. 16	18.7	42.8	27.5	11.4	4.9*	6.4	1.09	10.2	363 " "
May 17	18.7	35.1	30.1	12.0	5.8‡	6.2	1.12	10.8	1 yr., 60 days old.
Aug. 17	16.8	31.1	31.0	10.0	4.2‡	5.8	Not determined.	9.7	1 yr., 152 days old.
Sept. 10	17.1§	35.7	30.4	9.0	4.1‡	4.9	" "	10.3	Pregnant 18 days; 1 yr., 176 days old.
Oct. 11	17.5	34.1	29.5	10.8	5.4*	5.3	" "	10.4	Pregnant 49 days; 1 yr., 207 days old.
Nov. 16	17.6	38.8	28.6	9.9*	5.1*	5.1	" "	10.5*	Pregnant 85 days; 1 yr., 243 days old.
Dec. 10	18.2	41.0	28.8	10.0	4.5‡	5.5*	" "	11.1	Pregnant 109 days; 1 yr., 267 days old.
1918									
Jan. 11	16.4	35.1	28.3	9.4	4.4‡	5.0	" "	10.3	Pregnant 141 days; 1 yr., 299 days old.

Experiment 15. Heifer 214—Concluded.

Date.	Blood, total P (mg. per 100 gm.).	Blood corpuscles.	Corpuscles, total P (mg. per 100 gm.).	Plasma.					Remarks.
				Phosphorus.			Nitrogen (gm. per 100 cc.).	Calcium (mg. per 100 gm.).	
				Total (mg. per 100 gm.).	Lipoid (mg. per 100 gm.).	Inorganic (mg. per 100 gm.)			
1918		*vol. per cent*							
Feb. 8	18.8	37.8	28.7	12.2	3.9‡	8.3	Not determined.	10.0	Pregnant 169 days; 1 yr., 327 days old; phosphate since Jan. 19.
Mar. 20	17.1	36.7	28.4	9.9	4.4‡	5.5	" "	9.6	Pregnant 209 days; 2 yrs., 2 days old; no phosphate since Feb. 27.
May 2	15.6	37.8	24.1	9.9	5.9‡	4.0	" "	Not determined.	Pregnant 252 days; 2 yrs., 45 days old; phosphate since Mar. 29.

* One determination only.

† Check 1, 5.5; check 2, 5.5; check 3, 4.8; check 4, 5.3; checks 1, 2, and 4 used in calculating average.

‡ Calculated by subtracting inorganic from total.

§ Check 1, 14.8; check 2, 17.3; check 3, 16.9; check 4, 16.9; checks 2, 3, and 4 used in calculating average.

of alfalfa hay, 25 pounds of corn silage. January 19 to February 26, 1918, she was fed hay and grain on alternate days, and disodium phosphate was added to the grain, the manner of feeding being as follows. 1 day, 6 pounds grain (as above), but with 22 gm. of phosphorus as Na_2HPO_4 added to it; 25 pounds of corn silage; the next day, 8 pounds of alfalfa hay, 25 pounds of corn silage. From February 27 to March 27, this manner of feeding was continued, but the grain was fed without the phosphate and the silage was raised to 30 pounds.

THE PHYSICAL PROPERTIES AND CHEMICAL COMPOSITION OF HUMAN AMNIOTIC FLUID.

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An inquiry into the biological meaning of the human amniotic fluid demands, first of all, a sufficient knowledge of its physical properties and chemical composition, information which many investigators have attempted to gain. Prochownick (1) studied the sodium chloride, solid substances, and urea in the amniotic fluid; Schroeder (2) determined the quantities of solid substances, ash, and albumins; Farkas and Scipiades (3) inquired into its physical properties and into the quantity of sodium chloride contained in it; Jacqué (4) and Grünbaum (5) measured its freezing point. These reports, however, are defective either in the methods used, or in the statement of their examinations; accordingly, in many cases, the results are highly problematical. Döderlein (6) and Jacqué (4) performed some fairly exact analyses of amniotic fluid of mammalia, but their results cannot be applied directly to human amniotic fluid.

Physical Properties of Human Amniotic Fluid.

A study of the literature of the amniotic fluid may be said to establish the following facts: the fluid has a specific gravity varying from 1.002 to 1.028; a freezing point which varies from $\Delta = -0.45$ to $-0.5°$; it is always hypotonic against the blood of the maternal body and the fetus. On the basis of these facts, most investigators have concluded that the amniotic fluid is not a simple transudate, but a mixture of fetal urine with maternal transudate.

Experimental Material.

The urine of women in labor was removed through a Nelaton's catheter after disinfection of the vulva; a sterile dry cylindrical speculum was in-

troduced deep into the vagina, and the os uteri and its circumference were wiped with sterile gauze, thereby cleansing it entirely from blood, mucous particles, etc. The fetal membranes were pierced with a sharp instrument, and the amniotic fluid flowing out from the inside of the womb was collected in a clean glass flask, and preserved with a small quantity of toluene in an ice box. Thus obtained, the amniotic fluid is almost pure, since it has little chance to become impure, as it flows along the sides of the speculum, without touching the mucous membrane of the vagina or the skin of the vulva.

The amniotic fluid is slimy, yellowish white or pale yellow, and cloudy like soap water (rarely clear); it almost always contains much mucous flocks and has a peculiar odor. Because of its sliminess, the fluid is first filtered through fourfold gauze and later with suction. Thus prepared, the slightly cloudy liquor was used for the following experiments.

Specific Gravity.

With a 5 or 10 cc. pyknometer, the specific gravity of the amniotic fluid of women at the end of pregnancy was determined in twenty-three cases, and was found to average 1.0078. In six cases, the liquor was yellowish green, mixed with meconium, with a specific gravity varying from 1.0081 to 1.0136, averaging 1.0106; in the remaining seventeen cases, the liquor was yellowish white or yellow, and the specific gravity varied from 1.0046 to 1.0099, averaging 1.0069. Furthermore, in one case in the 5th month of pregnancy, in which artificial abortion was performed because of tuberculosis, the specific gravity of the fluid was 1.008, and in another case at the beginning of the 6th month, it was 1.0087. The admixture of meconium always causes an increase of the specific gravity. In one instance, in the first half of pregnancy, no evident difference was found.

The relation between the specific gravity and the freezing point is shown in Table I.

Freezing Point and Osmotic Pressure.

By use of Beckmann's cryoscopic apparatus, the freezing point of the amniotic fluid of twenty-two women at the end of pregnancy was determined. The value of Δ was found to vary from 0.46 to 0.565°, with an average of 0.504°. In sixteen cases, the amniotic fluid was yellowish or yellowish white, with an average freezing point of 0.504°; in the other six cases, the liquor was yellowish green, mixed with meconium, and had an average freez-

TABLE I.

No. of amniotic fluid.	Specific gravity.	Temperature of fluid.	Freezing point (Δ).	$C = \frac{\Delta}{1.86}$	Osmotic pressure in atmospheres (at 0°C).	New-born child.			Remarks.
						Sex.	Length.	Weight.	
		°C.	°C.				cm.	gm.	
1	1.0066	25	0.485	0.260	5.837	F.	49	2,500	
2	1.0072	21	—	—	—	M.	47	2,460	
5	1.0065	19	0.498	0.267	5.993	F.	50	2,500	
7	1.0076	18	0.480	0.258	5.777	M.	46	2,150	
8	1.0067	19	0.565	0.303	6.800	F.	50	3,000	
10	1.0083	19	0.563	0.302	6.776	M.	54	3,200	
12	1.0071	19	0.500	0.268	6.017	"	48	2,780	
13	1.0065	19	0.475	0.255	5.716	F.	49	3,000	
14	1.0066	19	0.500	0.268	6.017	"	47	3,300	
16	1.0073	19	0.490	0.263	5.897	M.	50	3,110	Asphyxia of second degree.
18	1.0065	19	0.460	0.247	5.536	F.	49	2,600	
19	1.0082	17	0.520	0.279	6.258	M.	53	3,050	
20	1.0075	17	0.525	0.282	6.318	"	49	2,500	
22	1.0066	18	0.512	0.275	6.162	F.	41	2,070	
23	1.0064	17	0.505	0.271	6.077	M.	52	3,420	
24	1.0074	17	0.500	0.268	6.017	"	49	2,970	
27	1.0046	18	0.495	0.266	5.957	F.	47	3,050	
Average..	1.0069		0.504	0.271	6.072	—	—	——	
4	1.0136	21	0.565	0.303	6.800	M.	46	2,000	Albuminuria, beriberi, still-born, mixed with meconium.
6	1.0123	18	0.518	0.278	6.234	"	50	2,950	With meconium.
9	1.0090	19	0.478	0.256	5.753	"	52	2,810	" "
11	1.0099	19	0.513	0.275	6.174	"	54	2,800	" "
17	1.0108	19	0.488	0.262	5.873	"	48	2,680	" "
21	1.0081	17	0.470	0.252	5.656	"	55	3,150	" "
Average..	1.0106		0.505	0.271	6.081	—	—	—	
191	1.0080	34	—	—	—				5th month.
227	1.0087	18	0.540	0.293	6.499				6th "

ing point of 0.505°. According to these results, it seems that the admixture of meconium has no marked influence upon the freezing point. The amniotic fluid of a pregnant woman at the beginning of the 6th month showed the freezing point Δ to be 0.54°.

From the formulas $P = 0.082\ CT$ (in which P = osmotic pressure, C = osmotic concentration, and T = absolute temperature) and $C = \frac{\Delta}{E}$ (in which Δ = the lowering of freezing point, and E = a constant), the osmotic pressure of the liquor at 0° was calculated (Table I). The values for the sixteen cases of pure amniotic fluid varied from 5.536 to 6.80 atmospheres, with an average of 6.072, while those of the six cases in which the fluid was mixed with meconium varied from 5.656 to 6.80, with an average of 6.081 atmospheres.

Specific Electrical Conductivity.

The resistance of twenty-three samples of amniotic fluid was measured by the usual method, and the specific electrical conductivity calculated from the formula $U = \frac{C}{W}$ (W = resistance, C = resistance capacity). The resistance capacity was determined by using a 0.1 N KCl solution. In seventeen cases, the amniotic fluid was yellowish and the specific electrical conductivity varied from 119.06×10^{-4} to 134.00×10^{-4}, with an average of 127.15×10^{-4}. In six cases, the fluid was yellowish green, mixed with meconium, and the specific electrical conductivity varied from 120.62×10^{-4} to 132.84×10^{-4}, with an average of 126.90×10^{-4}. The admixture of meconium seems to have no marked influence on the electrical conductivity.

Hydrogen Ion Concentration.

According to Hasselbalch's (7) method, a gas electrode was made with amniotic fluid, and connected with a calomel electrode (0.3377 volt), an accumulator whose electromotive force was previously determined in comparison with a cadmium standard cell, a resistance bridge, and a capillary electrometer; the electromotive force of the fluid was determined, and from this the H ion concentration was calculated. The results with the H ion concentration are shown in Table II.

In sixteen cases, the amniotic fluid was pure, and the H ion concentration varied from 0.2266×10^{-8} to 0.2648×10^{-7}, with an average of 0.1282×10^{-7}. In five cases, the liquor was yel-

TABLE II.

No. of amniotic fluid.	Specific electrical conductivity at 25°.	Reaction (litmus).	H ion concentration.	Temperature.	Remarks.
				°C.	
1	124.43×10^{-6}	Weakly alkaline.	0.4845×10^{-8}	25	
2	$128.90 \times$ "				
5	$128.90 \times$ "	Weakly alkaline.	$0.7189 \times$ "	19	
7	$127.84 \times$ "	" "	$0.9270 \times$ "	19	
8	$129.11 \times$ "	" "	$0.2266 \times$ "	18	
10	$122.11 \times$ "	Neutral.	$0.3207 \times$ "	19	
12	$130.55 \times$ "	"	$0.5251 \times$ "	19	
13	$124.64 \times$ "	"	0.2269×10^{-7}	19	
14	$125.34 \times$ "	Weakly alkaline.	$0.2536 \times$ "	19	
16	$128.89 \times$ "	Neutral.	$0.2062 \times$ "	19	
18	$119.09 \times$ "	Weakly alkaline.	0.9804×10^{-8}	17	
19	$134.00 \times$ "	" "	0.2583×10^{-7}	17	
20	$134.00 \times$ "	" "	0.5002×10^{-8}	17	
22	$133.22 \times$ "	" "	$0.8628 \times$ "	18	
23	$127.40 \times$ "	" "	0.2648×10^{-7}	18	
24	$122.54 \times$ "	Neutral.	$0.1449 \times$ "	18	
27	$120.69 \times$ "	"	$0.1432 \times$ "	18	
Average.	$127.15 \times$ "		$0.1282 \times$ "		
4	$132.84 \times$ "	Weakly alkaline.	0.8338×10^{-8}	21	Still-born, mixed with meconium.
6	$127.97 \times$ "	" "	$0.7189 \times$ "	19	Mixed with meconium.
9	$120.62 \times$ "	Neutral.	0.1453×10^{-7}	19	Mixed with meconium.
11	$130.14 \times$ "	"	0.6131×10^{-8}	19	Mixed with meconium.
17	$126.74 \times$ "	Weakly alkaline.	$0.8329 \times$ "	19	Mixed with meconium.
21	$123.14 \times$ "	Neutral.			Mixed with meconium.
Average.	$126.90 \times$ "		0.8903×10^{-8}		

lowish green, mixed with meconium, and the H ion concentration varied from 0.6131×10^{-8} to 0.1453×10^{-7}, with an average of 0.8903×10^{-8}. It is evident from these figures that the reaction of the amniotic fluid is slightly alkaline. In addition, the reaction was tested with litmus paper; in thirteen cases, the reaction was weakly alkaline, in nine cases it was neutral; in no case was the reaction acid.

Optical Activity.

The albumin was coagulated by adding a small quantity of sodium chloride, making the solution slightly acid with 1 per cent acetic acid, and heated on a water bath. After filtering, only one of the twenty-two samples examined showed any optical activity (−0.01°).

Summary.—(1) The specific gravity of pure amniotic fluid averages 1.0069 and never rises higher than 1.010. (2) The freezing point averages 0.504°; the difference between the greatest and the smallest amounts to nearly 0.1°. The osmotic pressure averages 6.072 atmospheres. (3) The specific electrical conductivity averages 127.15×10^{-4}, and corresponds nearly to that of a 0.1 N solution of KCl. (4) The average H ion concentration is 0.1282×10^{-7}; that is, slightly alkaline. (5) The reaction with litmus paper is either slightly alkaline or neutral. (6) It is nearly optically inactive.

Inorganic Components of Human Amniotic Fluid.

Farkas and Scipiades (3) found 0.444 to 0.58 per cent (average 0.507 per cent) sodium chloride in the amniotic fluid of five pregnant women. Jacqué (4) analyzed the amniotic fluid of eighteen sheep fetuses with a length of 2 to 49 cm. and found: insoluble ash 0.017 per cent, soluble ash, 0.82 per cent, sodium chloride 0.64 per cent, and total ash 0.84 per cent. He also analyzed the fluid of two swine and found: insoluble ash 0.024 to 0.030 per cent, soluble ash 0.74 to 0.76 per cent, sodium chloride 0.53 to 0.55 per cent, and total ash 0.77 to 0.78 per cent. Döderlein (6) performed exact analyses of the amniotic fluid of fifteen cows and found: Cl 0.358 per cent, NaCl 0.586 per cent, Na_2O 0.367 per cent, K_2O, 0.060 per cent, Ca 0.014 per cent, Mg 0.0038 per cent. Nauta (8) found 0.36 per cent chlorine, 1.44 per cent solid substances, and 0.59 per cent ash in the amniotic fluid of cows.

Quantitative Determination of Water, Solid Substances, Ash, and Organic Substances.

The filtered amniotic fluid was centrifuged for 30 minutes, freed from sediment, and filtered with suction. A known quantity of this filtrate, which in most cases was still a little cloudy, was evaporated on a water bath, and dried to constant weight

at 110°C., giving the solid substance. This was then ashed and separated into soluble and insoluble ash. The sum of the two is total ash; the organic substances and the water were obtained by difference. The results of the analyses are shown in Tables III to VI.

TABLE III.

Amniotic fluid.	Average.	High.	Low.
	Gm. per 100 cc. fluid.		
Water (15 cases)	99.49	99.76	99.29
Solid substances (26 cases)	1.24	1.53	1.02
Soluble ash (20 cases)	0.77	0.85	0.72
Insoluble ash (20 cases)	0.04	0.05	0.03
Total ash (20 cases)	0.81	0.90	0.76
Organic substances (20 cases)	0.45	0.72	0.26

TABLE IV.

No. of amniotic fluid.	Quantity of amniotic fluid.	Solid substances per 100 cc.	Water per 100 cc.	Remarks.
		A.		
	cc.	*gm.*	*gm.*	
1	20	1.11	99.55	
5	20	1.11	99.54	
7	15	1.27	99.49	
12	20	1.20	99.51	
13	20	1.14	99.51	
14	15	1.26	99.40	
Average.		1.18	99.50	
		B.*		
4	20	1.73	99.63	Mixed with meconium.
6	10	1.84	99.39	" " "
9	20	1.77	99.13	" " "
11	20	1.95	99.04	" " "
Average.		1.82	99.30	

* Excluded from the average.

TABLE V.

No. of amniotic fluid.	Quantity of amniotic fluid.	Solid substances.	Soluble ash.	Insoluble ash.	Total ash.	Organic substances.	Water.	Remarks.
		Gm. per 100 cc. fluid.						
	cc.							
16	25	1.19	0.75	0.04	0.79	0.40	99.54	
17	20	1.32	0.72	0.04	0.76	0.56	99.76	Mixed with a little meconium.
18	20	1.02	0.72	0.04	0.76	0.27	99.63	
19	20	1.53	0.77	0.04	0.81	0.72	99.29	
20	20	1.25	0.78	0.04	0.82	0.43	99.50	
22	15	1.23	0.80	0.04	0.84	0.39	99.43	
23	25	1.15	0.74	0.04	0.78	0.36	99.49	
24	20	1.35	0.75	0.04	0.78	0.57	99.39	
27	20	1.13	0.72	0.04	0.76	0.37	99.33	
29	15	1.41	0.81	0.04	0.86	0.55		
Average.		1.26	0.76	0.04	0.80	0.46	99.48	

TABLE VI.

No. of amniotic fluid.	Quantity of amniotic fluid.	Solid substances.	Soluble ash.	Insoluble ash.	Total ash.	Organic substances.	Remarks.
		Gm. per 100 cc. fluid.					
	cc.						
30	20	1.27	0.76	0.04	0.80	0.46	
35	20	1.24	0.79	0.03	0.82	0.42	
37	20	1.34	0.73	0.05	0.78	0.56	
39	20	1.16	0.78	0.05	0.83	0.33	
40	20	1.07	0.72	0.04	0.76	0.31	
41	20	1.42	0.84	0.04	0.88	0.54	
44	20	1.14	0.79	0.03	0.82	0.32	
47	20	1.50	0.76	0.04	0.79	0.70	Mixed with a little meconium.
49	20	1.22	0.80	0.04	0.84	0.38	
50	20	1.26	0.85	0.04	0.90	0.36	
Average.		1.26	0.78	0.04	0.82	0.44	

Quantitative Determination of Inorganic Components.

The soluble ash was dissolved in water; chlorine, sulfuric acid, phosphoric acid, potassium, sodium, calcium, and magnesium were determined. The insoluble ash was dissolved in hydrochloric acid, then diluted with water, and used for the determinations of sulfuric acid, phosphoric acid, calcium, and magnesium.

Chlorine was precipitated and weighed as silver chloride. Sulfuric acid was precipitated as barium sulfate. Phosphoric acid was determined in the aqueous solution or hydrochloric acid solution by Neumann's (9) method.

Potassium and Sodium.—Phosphoric acid and sulfuric acid were removed as insoluble barium salts; ammonium carbonate solution and ammonia were added to the filtrate to precipitate the excess barium; the filtrate was evaporated after adding a drop of hydrochloric acid; the ammonium carbonate and ammonium chloride were removed by heating, and the residue was weighed as total alkali chlorides. The quantity of potassium in these alkali chlorides was determined as potassium platinic chloride, and the quantity of sodium determined by difference.

Calcium.—The aqueous or hydrochloric acid solution was treated with liquid ammonia till it reacted strongly alkaline; it was then made acid with acetic acid, and the precipitate of iron salts filtered. The filtrate was warmed on a water bath and, after addition of ammonium oxalate solution, was further warmed until the calcium oxalate was entirely precipitated. The precipitate of calcium oxalate was heated and weighed as calcium oxide.

Magnesium.—The filtrate from the calcium oxalate was concentrated, ammonia added till it reacted strongly alkaline, and sodium citrate and sodium phosphate were added. The precipitate of calcium magnesium phosphate was converted into magnesium pyrophosphate and weighed.

Iron was determined by Neumann's (10) method.

A preliminary qualitative analysis indicated the presence in the soluble ash of amniotic fluid of chlorine, sulfuric acid, phosphoric acid, Na, K, Ca, and Mg, and in the insoluble ash of carbonic acid, sulfuric acid, phosphoric acid, Ca, Mg, and Fe.

The 200 cc. of amniotic fluid examined in Table IV, and those in Tables V and VI were quantitatively analyzed, with results as shown in Table VII.

For a fourth sample, 10, 20, and 170 cc. of amniotic fluid were taken respectively from three women—200 cc. in all— and for a fifth sample, 60,

TABLE VII.

	Amniotic fluid (200 cc.) (Table V).				Amniotic fluid (200 cc.) (Table VI).			
	Soluble ash (1.5653 gm.).	Insoluble ash (0.0822 gm.).	Total ash (1.5875 gm.).	Ash per 100 cc. fluid.	Soluble ash (1.5651 gm.).	Insoluble ash (0.0807 gm.).	Total ash (1.6458 gm.).	Ash per 100 cc. fluid.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Cl	0.7313	0	0.7313	0.3656	0.7377	0	0.7377	0.3688
NaCl*			1.2050	0.6025			1.2154	0.6077
SO_3	0.1056	Trace.	0.1056	0.0528	0.1490	Trace.	0.1490	0.0745
S	0.0422	"	0.0422	0.0211			0.0596	0.0298
P_2O_5	0.0048	0.0082	0.0130	0.0065	0.0027	0.0094	0.0121	0.0060
P			0.0056	0.0028	0.0011	0.0041	0.0052	0.0026
Na_2O	0.7531	0	0.7531	0.3765	0.7661	0	0.7661	0.3830
Na	0.5587	0	0.5587	0.2793	0.5685	0	0.5685	0.2842
K_2O	0.0489	0	0.0489	0.0244	0.0464	0	0.0464	0.0232
K	0.0408	0	0.0408	0.0204	0.0385	0	0.0385	0.0192
CaO	0.0030	0.0314	0.0344	0.0172	0.0024	0.0302	0.0326	0.0163
Ca	0.0021	0.0224	0.0245	0.0122	0.0017	0.0215	0.0232	0.0116
MgO	Trace.	0.0008	0.0008	0.0004	0.0010	0.0002	0.0012	0.0006
Mg	"	0.0004	0.0004	0.0002	0.0006	0.0001	0.0007	0.0003

* The quantity of NaCl was calculated from the quantity of Cl.

40, and 100 cc. were taken. After complete evaporation and conversion into ash, quantitative analyses were performed, with results as in Table VIII.

Examining the above figures, we find that the only marked variation is in the amount of sulfuric acid.

To find the relations of the albumins in amniotic fluid to sulfuric acid and other ash, 400 cc. of the fluid obtained from fourteen cases were divided into two equal parts. The one part (A) was dried, ashed, dissolved in water, and diluted up to 200 cc., of which 20 cc. were used for the quantitative determination of sulfuric acid, 50 cc. for phosphoric acid, and 130 cc. for Ca and Mg. The other part (B) was diluted with nine volumes of water, and the albumins were entirely precipitated with common salt and colloidal ferric hydroxide according to Rona and Michaelis (11) and Oppler and Rona. The precipitate was mixed with 500 cc. of water and filtered; this treatment was repeated two more

TABLE VIII.

	I. Amniotic fluid (200 cc.).				II. Amniotic fluid (200 cc.).	
	Soluble ash (1.5385 gm.).	Insoluble ash (0.0547 gm.).	Total ash (1.5932 gm.).	Ash per 100 cc. fluid.	Total ash.	Ash per 100 cc. fluid.
	gm.	gm.	gm.	gm.	gm.	gm.
Cl	0.7486	0	0.7486	0.3743	—	—
NaCl	—	—	1.2336	0.6168	—	—
SO_3	0.0661	0.0027	0.0688	0.0344	0.0576	0.0288
S	—	—	0.0274	0.0137	—	0.0115
P_2O_5	0.0042	0.0112	0.0154	0.0077	0.0130	0.0065
P	0.0018	0.0048	0.0066	0.0033	0.0056	0.0028
Na_2O	0.7729	0	0.7729	0.3864	—	—
Na	0.5737	0	0.5737	0.2868	—	—
K_2O	0.0444	0	0.0444	0.0222	—	—
K	0.0372	0	0.0372	0.0186	—	—
CaO	—	—	—	—	0.0321	0.0160
Ca	—	—	—	—	0.0229	0.0114
MgO	—	—	—	—	0.0019	0.0009
Mg	—	—	—	—	0.0012	0.0006

times. The filtrates were then combined, evaporated, after the addition of a little acetic acid, ashed, and analyzed. In the second part (B) the amount of sulfuric acid evidently was decreased, and the quantity of magnesium also a little diminished. From these facts we may conclude that a part of the sulfuric acid in the ash of amniotic fluid is produced from the sulfur of albumins.

TABLE IX.

	Amniotic fluid (200 cc.) (A).	Ash per 100 cc. fluid.	Amniotic fluid (200 cc.) (B).	Ash per 100 cc. fluid.
	gm.	gm.	gm.	gm.
SO_3	0.0518	0.0259	0.0227	0.0113
S	—	0.0103	—	0.0045
P_2O_5	0.0142	0.0071	—	—
P	—	0.0030	—	—
CaO	0.0252	0.0126	0.0268	0.0134
Ca	—	0.0090	—	0.0096
MgO	0.0008	0.0004	0.0002	0.0001
Mg	—	0.0002	—	Trace.

To discover the relations of the phosphoric acid in the ash of amniotic fluid to albumins and other organic substances in the liquor, samples of the fluid from two women were divided into two parts. One part (A) was used for the quantitative determination of phosphoric acid. The other part (B) was freed from albumins by coagulation; the organic phosphorus substances were removed from the filtrate by extraction with ether, and the phosphoric acid was determined according to Neumann. The two results were nearly identical.

TABLE X.

	I. Amniotic fluid (80 cc.).		II. Amniotic fluid (160 cc.).	
	A (40 cc.).	B (40 cc.).	A (60 cc.).	B (100 cc.).
	Gm. per 100 cc. fluid.		Gm. per 100 cc. fluid.	
P_2O_5	0.0049	0.0050	0.0045	0.0042
P	0.0021	0.0020	0.0019	0.0018

In order to decide whether calcium or magnesium combined with albumins, 100 cc. of amniotic fluid from two cases were divided into two equal parts; one part (A) was used for the quantitative determination of Ca and Mg; the other part (B) was freed from albumins with colloidal ferric hydroxide, and used for the same determination. The second half (B) was but little reduced in the quantity of Mg.

TABLE XI.

	Amniotic fluid (100 cc.).			
	A (50 cc.).		B (50 cc.).	
	gm.	Gm. per 100 cc. fluid.	gm.	Gm. per 100 cc. fluid.
CaO	0.0082	0.0164	0.0077	0.0154
Ca	0.0058	0.0116	0.0055	0.0110
MgO	0.0002	0.0004	Trace.	Trace.
Mg	0.0001	0.0002	"	"

To decide whether the amniotic fluid contains ether sulfuric acid, 100 cc. of the fluid were collected from four cases, the albumins were precipitated with colloidal ferric hydroxide, and filtered,

the whole volume being 1,000 cc. From the filtrate, 900 cc. (corresponding to 90 cc. of amniotic fluid) were evaporated to 85 cc.; 15 cc. of dilute hydrochloric acid and 15 cc. of 5 per cent barium chloride solution were added, and barium sulfate was filtered. The weight of barium sulfate produced here represents the inorganic sulfuric acid. The filtrate of barium sulfate was boiled on a water bath until the newly produced barium sulfate was entirely precipitated; then from its weight, the weight of ether sulfuric acid was calculated, as shown in Table XII.

TABLE XII.

	Amniotic fluid (100 cc.).	
		gm.
Inorganic sulfuric acid.....	SO_3	0.0279
Ether " "	SO_3	0.0006
Total " "	SO_3	0.0285

In two experiments 30 cc. of amniotic fluid from two cases each, were reduced to ash, dissolved in hydrochloric acid, and the quantity of iron was determined by Neumann's method. The results are given in Table XIII.

TABLE XIII.

	Amniotic fluid (30 cc.).		Amniotic fluid (30 cc.).	
	gm.	Gm. per 100 cc. fluid.	*gm.*	Gm. per 100 cc. fluid.
Fe.....	0.00248	0.0082	0.00266	0.0088
Fe_2O_3.....	0.00709	0.0236	0.0076	0.0253

Furthermore, in one case each of the 5th, 6th, 8th, and 9th months of pregnancy the quantity of sodium chloride was determined by Volhard's (12) method (Table XIV).

These values show that there is no marked difference to be found in the quantity of sodium chloride at different periods of pregnancy.

Summary.—(1) The amniotic fluid of women at the end of pregnancy always contains Cl, carbonic acid, sulfuric acid, phos-

TABLE XIV.

No. of amniotic fluid.	Cl	NaCl	Remarks.
	Gm. per 100 cc. fluid.		
118	0.3939	0.65	35th week of pregnancy.
123	0.3636	0.6	Middle of 8th month, still-born, weight of fetus, 1,800 gm., congenital syphilis (?).
191	0.3211	0.53	5th month, artificial abortion for consumption.
227	0.3939	0.65	Beginning of 6th month.

phoric acid, Na, K, Ca, Mg, and Fe. (2) The amounts of Cl, phosphoric acid, K, Na, Ca, and Fe are nearly constant. (3) The greatest part of the inorganic salts is sodium chloride, which averages 75.2 per cent of the total ash. (4) A part of the sulfuric acid in the ash comes from the sulfur of albumins and a very small part comes from ether sulfuric acid. Therefore, the quantity of sulfuric acid in the ash depends on the quantity of albumins. (5) The average quantities of the inorganic components are shown in Table XV.

TABLE XV.

Amniotic fluid (100 cc.).

Inorganic components.	Average gm.	Inorganic components.	Average gm.
Water	99.4880	P	0.0026
Solid substances	1.2425	Na_2O	0.3819
Organic substances	0.4504	Na	0.2834
Soluble ash	0.7690	K_2O	0.0232
Insoluble ash	0.0407	K	0.0194
Total ash	0.8098	CaO	0.0157
Cl	0.3695	Ca	0.0111
NaCl	0.6090	MgO	0.0005
SO_3	0.0432	Mg	0.0003
S	0.0172	Fe_2O_3	0.0244
P_2O_5	0.0061	Fe	0.0085

d-Lactic Acid and Sugars in Human Amniotic Fluid.

d-Lactic Acid.

Zweifel (13) examined the urine of eclampsia patients and found an increase of ammonia and the existence of *d*-lactic acid. In the same cases he confirmed the existence of a certain quantity of *d*-lactic acid in the blood of maternal bodies, the blood of the umbilical cord, and the placenta. Ten Doeschate (14) also states that in eclampsia cases the *d*-lactic acid is always present in the blood and urine of maternal bodies, in the blood of the umbilical cord, and in the placenta. Because, however, in one case of normal confinement, a quantity of *d*-lactic acid was found in maternal blood corresponding to 0.029 per cent zinc lactate, in placenta corresponding to 0.119 per cent zinz lactate, Ten Doeschate concluded that Zweifel's assertion cannot be adequate, *i.e.*, the *d*-lactic acid is not the cause of eclampsia, but the convulsion in eclampsia provokes the accumulation of lactic acid. The relations between lactic acid in the blood and urine and obstetrical diseases were the subjects of the foregoing investigations; the presence of lactic acid in the amniotic fluid is examined for the first time in the following experiments.

Method.

Amniotic fluid was evaporated on a water bath to one-tenth its volume then poured into ten parts alcohol, and extracted. The alcoholic extract was concentrated, the residue dissolved in a small quantity of water, phosphoric acid added until the solution was strongly acid, and the solution extracted in a Suto extraction apparatus with ether for 24 hours. The brown residue from the ethereal extract was dissolved in water, treated with freshly prepared lead carbonate, heated on a water bath for nearly 30 minutes, and filtered. The filtrate was freed from lead by H_2S, the H_2S removed by vacuum distillation, zinc oxide added to the condensed solution, the mixture boiled on a water bath for 30 minutes, and filtered. The filtrate was decolorized by blood charcoal and the clear solution thus obtained was evaporated until the zinc lactate began to crystallize, when a little alcohol was added. As the white crystals of zinc lactate were not yet quite pure, they were washed several times with absolute alcohol, dissolved in a small quantity of hot water and brought again to crystallization.

It was then assured that the white crystals thus prepared were no other than those of zinc *d*-lactate by (1) examination of crystal shape, (2) Uffelmann's reaction, (3) Fletcher and Hopkins' reaction, (4) measurement of the specific rotation, (5) quantitative determination of the water of crystallization, (6) elementary analysis.

Experiment 1.—1,000 cc. of clean amniotic fluid collected from fourteen healthy women in labor, were treated as above, giving 0.9 gm. of zinc salt, colorless needles, or rhombic prisms, giving a positive Uffelmann's reaction and Fletcher and Hopkins' reaction.

$$[\alpha]_D^{15^\circ} = \frac{-0.4 \times 100}{2.506 \times 2} = -7.98^\circ.$$

0.3359 gm. salt lost 0.0433 gm. H_2O on drying at 110° to constant weight.
0.2045 " dry salt gave 0.0679 gm. ZnO.
0.0768 " " " " 0.0834 " CO_2 and 0.027 gm. H_2O.

Experiment 2.—1,000 cc. of yellowish green amniotic fluid collected from twenty cases and mixed with a small quantity of meconium, gave 1 gm. of zinc salt.

$$[\alpha]_D^{20^\circ} = \frac{-0.58 \times 100}{3.646 \times 2} = -7.95^\circ.$$

0.1567 gm. salt lost 0.0204 gm. H_2O.
0.211 " dry salt gave 0.0707 gm. ZnO.

Experiment 3.—2,000 cc. of amniotic fluid obtained from a case of acute hydramnios at the end of the 8th month of pregnancy, gave 1.917 gm. of zinc salt.

$$[\alpha]_D^{20^\circ} = \frac{-0.66 \times 100}{4.084 \times 2} = -8.08^\circ.$$

0.5143 gm. salt lost 0.0668 gm. H_2O.
0.112 " dry salt gave 0.0374 gm. ZnO.
0.2054 " " " " 0.073 gm. H_2O and 0.2187 CO_2.

From the foregoing results it is possible to conclude that *d*-lactic acid is a constant component of human amniotic fluid.

TABLE XVI.

Zinc salt prepared from human amniotic fluid.				Zinc *d*-lactate.
Experiment.	1	2	3	
Crystals.	Needle-shaped or rhombic prisms.			
Specific rotation $[\alpha]_D^{15^\circ}$	−7.98°	−7.95°	−8.08°	−8.6°
	per cent	*per cent*	*per cent*	*per cent*
Water of crystallization	12.89	13.01	12.98	12.86
Zn	26.65	26.91	26.83	26.86
C	29.62	—	29.71	29.54
H	3.86	—	3.95	4.13

Quantitative Determination of the d-Lactic Acid in Human Amniotic Fluid.

According to Yoshikawa's (15) method, a known quantity of amniotic fluid, after filtering and centrifuging, was evaporated on a water bath, extracted with about ten parts of alcohol, and filtered. The sediment was washed several times with alcohol, the wash alcohol and the alcoholic extract were combined and concentrated, the residue was dissolved in water made strongly acid by the addition of phosphoric acid, and extracted in a Suto extraction apparatus with ether for 72 hours. The residue of the ethereal extract was dissolved in water, lithium carbonate added, and the mixture heated on a water bath for a half hour and filtered. After decolorizing with blood charcoal, the solution was evaporated and made up to 20 cc. From the observed rotatory angle the amount of lithium *d*-lactate of *d*-lactic acid in the amniotic fluid was calculated.

With the same method, *d*-lactic acid was determined in ten cases which varied from 0.1355 per cent to 0.0336, with an average of 0.0726 per cent.

TABLE XVII.

No. of experiment.	No. of amniotic fluid.	Quantity of amniotic fluid.	Temperature.	Rotary angle (α).	Quantity of lithium lactate.	Quantity of *d*-lactic acid.		Remarks.
							Gm. per 100 cc. fluid.	
		cc.	°*C.*		*gm.*	*gm.*		
I	63	60	12	−0.03°	0.02098	0.02017	0.0336	End of 10th month, mixed with a little meconium.
II	92	60	12	−0.050°	0.03506	0.03371	0.0561	10th month.
III	95	60	12	−0.035°	0.02469	0.02374	0.0395	10th "
IV	96	120	12	−0.100°	0.07012	0.06742	0.0561	10th "
V	97	120	12	−0.130°	0.09134	0.08782	0.0761	10th "
VI	98	60	12	−0.055°	0.03850	0.03700	0.0617	Mixed with meconium, 10th month.
VII	116	100	25	−0.100°	0.07010	0.06740	0.0674	10th month.
VIII	118	100	25	−0.190°	0.13407	0.12890	0.1289	35th week of pregnancy.
IX	122	100	25	−0.110°	0.07720	0.07430	0.0743	10th month.
X	128	100	25	−0.200°	0.14090	0.13550	0.1355	10th "
Average							0.0726	

Sugars.

Gürber and Grünbaum (16) found some fruit sugar in the amniotic fluid of cows, sheep, and swine, but could not find even a trace of sugars in the fluid of ten women examined in the same way. Grünbaum (17) states that the amniotic fluid of cats and dogs is free from fruit sugar. However, Ludwig (18) found 0.3 gm. of sugar in 4,000 cc. of amniotic fluid obtained by artificial puncture of fetal membranes of a diabetes patient, whose urine contained about 3.8 per cent sugar.

In order to decide whether human amniotic fluid is surely free from sugars, an aqueous solution, after being freed from lactic acid, was neutralized with calcium carbonate, and filtered. The voluminous precipitate of calcium phosphate was then mixed with water in a porcelain mortar and filtered again by suction. The filtrates were combined and concentrated to 100 cc. and used for the following examinations: Trommer's and Nylander's reactions were negative; the phenylhydrazine reaction was negative; and finally, the solution was optically inactive. The results, therefore, of these experiments confirm the statements of Gürber and Grünbaum that sugars are not a constant component of human amniotic fluid.

Summary.—(1) The *d*-lactic acid is a constant component of human amniotic fluid and amounts to 0.0726 per cent on an average. (2) Sugars are not to be found in human amniotic fluid.

Allantoin and Organic Bases in Human Amniotic Fluid.

According to Gusserow and Hermann, the urine of healthy bodies always contains a small quantity of allantoin, while the urine of pregnant women contains more. Wiechowski (19), however, states that he could find no allantoin in human urine, in 200 cc. of urine of a 1½ months old suckling baby, nor in the urine of a new-born child at its first day; furthermore, allantoin was absent in the amniotic fluid of two pregnant women. It is still an open question whether allantoin is a component of human amniotic fluid; the amino-acids or organic bases of human amniotic fluid have not been investigated.

In the present investigation, 3,064 cc. of amniotic fluid were precipitated with basic lead acetate and filtered. The filtrate was freed from lead, concentrated *in vacuo* at 50° to about 150 cc., and filtered. The filtrate was diluted with water to 200 cc., concentrated sulfuric acid added until the latter amounted to 5 per cent in volume, and the solution filtered. The filtrate was treated with 20 per cent phosphotungstic acid solution and, after standing for a night, filtered, giving a reddish white precipitate (A),

used to test for organic bases, and a filtrate (B), used to test for amino-acids and allantoin.

Organic Bases.—The precipitate (A) was washed several times with 5 per cent sulfuric acid, then mixed with barium hydroxide in a porcelain mortar, and diluted with water. Carbon dioxide was passed into this mixture, the barium carbonate filtered, and the filtrate distilled *in vacuo* to 50 cc.; addition of dilute nitric acid and silver nitrate gave a small quantity of brown precipitate (I), purine bases, and a filtrate (II).

Purine Bases.—Precipitate I gave a negative test for purine bases.

Hexone Bases and Choline.—Filtrate II was divided into two portions with silver nitrate and barium hydroxide; the resulting precipitate (III) was washed several times with dilute barium hydroxide and used for the preparation of histidine and arginine, while the filtrate (IV) was used to test for choline and lysine. Using the ordinary tests and methods of separation, histidine was demonstrated by a positive Pauly diazo reaction; a picrate was obtained as fine yellow, silken needles, where one would expect to find arginine, but its melting point was 182°. Lysine was isolated as the picrate, orange-yellow needles, melting at 252°. Choline was absent.

Allantoin.—Allantoin was isolated from filtrate B according to Wiechowski (20). (The filtrate from the mercury acetate was used to prove the presence of amino-acids.) The material obtained from the mercury acetate gave a positive Eppinger (21) glyoxylic acid reaction, and gave oxalic acid when heated with 15 per cent sodium hydroxide (Salkowski, 22). Thus, while pure crystals of allantoin could not be isolated, a very small quantity of allantoin probably is to be found in human amniotic fluid.

Amino-Acids.—The filtrate from the mercury acetate-allantoin precipitate was freed of Hg and found to contain 0.3 gm. N. The presence of amino-acids was tested for according to Ignatowski (23), but with negative results.

Summary.—The results of the experiment, which was performed with 3,000 cc. of amniotic fluid are as follows: (1) Neither purine bases, choline, nor monoamino-acids are to be found in amniotic fluid. (2) Histidine, lysine, and allantoin are all present in very small quantity. (3) The existence of arginine is still undecided.

Albumins, Urea, Uric Acid, Creatine, and Cholesterol in Human Amniotic Fluid.

Albumins.

Koettnitz (24) found propeptone in the amniotic fluid in two cases in pregnancy of 2¼ and 2½ months, respectively; peptone and propeptone in four cases in the 10th month, and peptone

alone in one case in the 10th month; from these facts he concluded that the amniotic fluid is used as nourishment for the fetus at the beginning of pregnancy, and has the same use, though in much less degree, at the end of pregnancy. Weyl (25) confirmed the presence of mucin in the amniotic fluid of two women in hydramnios and determined the quantities as 0.1 and 0.2 per cent.

Experiment 1.—37 cc. of amniotic fluid were centrifuged, filtered by suction, treated with an equal volume of a saturated solution of ammonium sulfate, and filtered after standing over night. The filtrate heated on a water bath, gave a heavy precipitate; the filtrate of this precipitate gave no biuret reaction. The precipitate, obtained by the addition of ammonium sulfate solution, was washed several times with a half saturated solution of ammonium sulfate, until its filtrate gave no more albumin reactions; when dissolved in a small quantity of water and heated on a water bath at more than 70°, the solution became white and cloudy; when boiled and filtered, the transparent filtrate showed a very weak biuret reaction.

Experiment 2.—60 cc. of nearly clear and yellowish amniotic fluid were diluted with an equal volume of water and filtered by suction. When mixed with a few drops of acetic acid, the filtrate showed a white cloudiness, which could not be redissolved by the addition of excess acetic acid. After standing over night, the slight precipitate was filtered and washed with very dilute acetic acid. The precipitate, boiled with dilute sulfuric acid, gave a solution with positive Trommer's reaction. The filtrate of the precipitate produced by the addition of acetic acid was neutralized with sodium carbonate, and upon adding an equal volume of a saturated solution of ammonium sulfate, gave a cloudiness. The filtrate was heated on a water bath and filtered from the coagulated albumins, but this filtrate gave no sign of biuret reaction.

The precipitate produced by the addition of ammonium sulfate was washed with a half saturated solution of ammonium sulfate, dissolved in a small quantity of water and heated on a water bath at more than 70° when the solution became white and cloudy. When boiled and filtered, the transparent filtrate still showed a very weak biuret reaction.

Experiment 3.—According to Hohlweg and Meyer (26), a mixture of a volume of 1 per cent acetic acid with an equal volume of 5 per cent monopotassium phosphate solution was added to 80 cc. of amniotic fluid until it reacted acid against Congo red paper, but neutral against litmus paper; then an equal volume of a saturated sodium chloride solution was added; this mixture was boiled on a water bath to coagulate the albumins, and filtered. The filtrate obtained thus gave no biuret reaction.

In Experiment 2, the appearance of the glucoprotein, which was produced by the addition of acetic acid and was not soluble

in excess acetic acid, confirmed the existence of mucin in amniotic fluid; Experiment 1 demonstrated that albumin and a little globulin were present in amniotic fluid, but that no peptones were present. Experiment 3 also demonstrated that peptones were absent. But whether the very weak biuret reaction in the filtrate of the coagulable albumins in Experiments 1 to 3 was due to the presence of albumoses could not be determined.

Quantitative Determination of Coagulable Albumins.

Prochownick (1) found 0.71 to 0.06 per cent coagulable albumins in the amniotic fluid of eleven women; Ahlfeld (27) states that the amniotic fluid of seven out of twenty women contained 25 to 50 volume per cent albumins, and from this fact he argued that amniotic fluid must be a nourishment for the fetus; however, his argument is not adequate as the following results of other authors indicate. According to Schroeder (2), the amount of albumins in the amniotic fluid stands in a close relation to the quantity of hemoglobin in the maternal blood; in 50 cases it amounted to 0.03 to 0.308 per cent. In the mature fetus, it averaged 0.1681 per cent, and 0.1578 per cent in the immature fetus. In Döderlein's (6) experiments with fifteen cows, the quantity of albumins in the amniotic fluid amounted to 0.042 to 0.455 per cent; it increased gradually as the pregnancy proceeded. Jacqué found 0.023 to 0.058 per cent albumins in the amniotic fluid of the sheep fetus, when less than 14 cm. in length, and 0.1 per cent albumins when over 30 cm. in length.

In the present experiments, human amniotic fluid was first filtered through fourfold gauze, then centrifuged, and filtered by suction. Thus prepared, the fluid was diluted with three parts of water, sodium chloride added to make 1 per cent, the solution made weakly acid with 1 per cent acetic acid, and boiled on a water bath. This completely coagulated the albumins which were filtered through a tared ashless filter, washed with warm water, then with alcohol, and finally with ether. Thus treated, the albumins were dried on the filter at 110° and weighed. Since the coagulated albumins thus obtained usually contained more or less ash, this ash was determined, and from it the weight of pure coagulable albumins calculated. The value obtained by the above method is the sum of the albumin, globulin, and mucin, but this may be considered coagulable albumins in a narrow sense, since the quantity of mucin in the amniotic fluid is very small.

The quantity of the coagulable albumins in the amniotic fluid of fifteen women at the end of pregnancy varied from 0.092 to

0.421 per cent, with an average of 0.226 per cent. In two cases of the 5th and 6th months of pregnancy, respectively, the amount of coagulable albumins is not markedly different from the amount at the end of pregnancy. The results of these experiments are shown in Table XVIII.

TABLE XVIII.

No. of experiment.	No. of amniotic fluid.	Quantity of amniotic fluid.	Coagulable albumin.		Remarks.
				Gm. per 100 cc. fluid.	
		cc.	gm.		
1	83	10	0.0421	0.421	
2	85	5	0.0149	0.299	
3	86	10	0.0160	0.160	
4	87	10	0.0229	0.229	
5	89	10	0.0277	0.277	
6	90	10	0.0259	0.259	
7	91	10	0.0149	0.149	
8	92	5	0.0046	0.092	
9	95	10	0.0215	0.215	
10	96	10	0.0352	0.352	
11	97	10	0.0115	0.115	
12	104	10	0.0131	0.131	
13	106	10	0.0190	0.190	
14	114	10	0.0265	0.265	
15	115	10	0.0238	0.238	
Average.				0.226	
16	191	10	0.0384	0.384	5th month, artificial abortion for consumption.
17	227	10	0.0416	0.416	Beginning of 6th month.

Quantitative Determination of Urea and Distribution of Nitrogen.

Although Scherer, Mack, and Calberg denied the existence of urea in the amniotic fluid, Woeler discovered it by exact investigation. Later, other investigators have given the following values: Funke, 0.38 per cent; Letzmann, 0.05 per cent; Majewski, 0.34 to 0.42 per cent; Beale (28), 0.35 per cent; Siewert (29), 0.0352 per cent; Winckel (30), 0.086 to 0.42 per cent; Gusserow (31) 0.14 to 0.35 per cent. Prochownick (7) determined the quantity of urea as nitric urea in two cases in the first half of pregnancy and also in eleven cases at the end of pregnancy, and found 0.0155 to 0.034 per cent; since the amount of urea was small at the beginning and greater at the end

of pregnancy, he concluded that urea in the amniotic fluid is secreted from the skin and the kidneys of the fetus, and that the fluid itself is a product of the fetus. Schöndorff (32) stated that the amount of urea in the amniotic fluid (0.0611 per cent) is about the same as that in the blood and the milk.

In the present investigations, the ammonia was determined by the method of Brugsch and Schittenhelm (33); Marshall's (34) method was used for the determination of urea, and Kjeldahl's for the total nitrogen. The albumin N was determined in the precipitate obtained with colloidal ferric hydroxide. The amount of ammonia varied from 0.0051 to 0.0013 per cent, with

TABLE XIX.

No. of experiment.	No. of Amniotic fluid.	Ammonia.	Urea.	Total N.	Albumin N.	Rest N.	Ammonia N.*	Urea N.	Remaining N.	Remarks.
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
1	112	0.0040	0.0276							Mixed with meconium.
2	113	0.0023	0.0570							
3	115	0.0015	0.0519							
4	117	0.0023	0.0666							Mixed with meconium.
5	122	0.0017	0.0504							
6	132	0.0013	0.0297							
7	143	0.0044	0.0204							Twins.
8	211	0.0028								
9	219	0.0051								
10	227	0.0017	0.0195	0.1036	0.0868	0.0168	0.0014	0.0091	0.0063	
11	232	0.0034	0.0300							
12	237	0.0051	0.0180	0.0588	0.0441	0.0147	0.0042	0.0084	0.0021	
13	243	0.0025	0.0195	0.0518	0.0336	0.0182	0.0021	0.0091	0.0070	
14	247	0.0034	0.0255							
15	248	0.0029	0.0187	0.0770	0.0635	0.0135	0.0024	0.0087	0.0024	
16	250	0.0029	0.0174	0.0840	0.0672	0.0165	0.0024	0.0061	0.0083	
Average.		0.0029	0.0323	0.0750	0.0590	0.0159	0.0025	0.0083	0.0052	

* The ammonia N corresponds to 15.7 per cent of the so called rest N, and the urea N corresponds to 52.2 per cent of the rest N.

an average of 0.0029 per cent; the urea varied from 0.0666 to 0.0174 per cent, with an average of 0.0323 per cent. Five experiments on the distribution of N gave average values of: albumin N, 0.059 per cent; rest N, 0.0159 per cent; ammonia N, 0.0025 per cent; urea N, 0.0083 per cent. These results are seen in Table XIX.

Uric Acid.

653 cc. of amniotic fluid were freed from coagulable albumins by boiling with dilute acetic acid, and then by adding 5 per cent monopotassium phosphate solution and boiling. The filtrate was then tested for uric acid by Schroeder's (35) method. The resulting silver compound was freed from silver, the residue extracted with a dilute sodium carbonate solution, the solution treated with acetic acid, and evaporated. After a day, some amorphous reddish sediment separated; this was insoluble in cold water, but easily dissolved upon warming and separated again upon cooling. A part of this sediment gave a marked murexide reaction, but there was not enough for an elementary analysis. A second experiment gave negative results. Uric acid in small quantity is contained in the amniotic fluid during confinement.

Creatine and Creatinine.

Gönner (36) states that creatine and creatinine should be contained in the amniotic fluid, but that they have not been secured experimentally.

In one experiment, the filtrate from the uric acid precipitate (silver and magnesia) was freed from silver by hydrochloric and nitric acids and used for the preparation of creatine according to Neubauer and Salkowski's (37) method, with negative results, neither creatine nor creatinine being found. In another experiment, 420 cc. of pure amniotic fluid, treated in the same way, showed the presence of creatine but creatinine was absent. Amniotic fluid during confinement contains creatine in very small quantity, but no creatinine.

Hippuric Acid.

Until the present experiments, no investigation has been made for hippuric acid as a component of the amniotic fluid.

526 cc. of amniotic fluid were precipitated with lead acetate, the lead in the filtrate was removed with H_2S, the filtrate concentrated *in vacuo* to a syrup, and extracted with alcohol; the residue was dissolved in a small quantity of hot water, acidified with dilute hydrochloric acid and extracted with acetic ether. This extract was washed with water, and, upon evaporation, gave some brown residue which was extracted with petroleum ether. The insoluble part contained no hippuric acid; two other similar experiments were likewise negative. Hippuric acid is not a component of human amniotic fluid.

Cholesterol.

Gönner (36) states that the amniotic fluid contains cholesterol, but no experimental investigation of it could be found in the literature.

For these experiments, 905 cc. of amniotic fluid were extracted with ether in a Suto extraction apparatus for 48 hours, the extract was saponified with dilute alcoholic potassium hydroxide, the residue treated with 20 cc. of hot water, and after cooling, extracted with ether three times. The residue was extracted with petroleum ether, and the soluble portion dissolved in a mixture of ether and alcohol, and the solution allowed to evaporate spontaneously. White, mica-like, glossy, squamiform crystals were obtained; examined under a microscope, they were colorless, very thin, rhombic or irregularly square tablets, sometimes broken at a corner, and piled closely one on top of another. They melted at 146°, were optically active, and gave positive Salkowski's, Liebermann and Burchard's, and Schiff's reactions. From this, it is evident that the substance obtained by extraction with ether from human amniotic fluid is cholesterol, and the cholesterol is beyond a doubt a component of human amniotic fluid.

Summary.—(1) The human amniotic fluid at the end of pregnancy always contains coagulable albumins (an average of 0.226 per cent, inclusive of mucin); albumin is its greatest constituent, but globulin is present in traces; the amount of albumins in the amniotic fluid in the first half of pregnancy is not remarkably different from that at the end. (2) Mucin is also contained, but in quantity too small to determine; peptone and albumoses are

not found. (3) Ammonia and urea are constant components; the amount of ammonia is, on an average, 0.0029 per cent; the amount of urea, on an average, is 0.0323 per cent, and both together furnish about 70 per cent of the so called rest N. (4) Uric acid and creatine, in very small amounts, are present, but neither creatinine nor hippuric acid is found. (5) Cholesterol is a component of human amniotic fluid.

BIBLIOGRAPHY.

1. Prochownick, L., *Arch. Gynäk.*, 1877, xi, 304, 561.
2. Schroeder, R., *Arch. Gynäk.*, 1891, xxxix, 306.
3. Farkas, G., and Scipiades, E., *Arch. ges. Physiol.*, 1903, xcviii, 577.
4. Jacqué, L., Mém. couron et autres mém. publ. par l'Acad. roy. des sc., des let. et des beaux-arts de Belg., 63, 117.
5. Grünbaum, D., *Deutsch. med. Woch.*, 1905, xxxi, 1676.
6. Döderlein, A., *Arch. Gynäk.*, 1890, xxxvii, 141.
7. Hasselbalch, K. A., *Biochem. Z.*, 1911, xxx, 317.
8. Nauta, J. S., Dissertation, Bern, 1905.
9. Neumann, A., *Z. physiol. Chem.*, 1902–03, xxxvii, 129.
10. Neumann, *Z. physiol. Chem.*, 1904–05, xliii, 33.
11. Michaelis, L., and Rona, P., *Biochem. Z.*, 1908, viii, 356. Oppler, B., and Rona, P., *Biochem. Z.*, 1908, xiii, 121.
12. Cf. Volhard in Hoppe-Seyler, F., Handb. physiol.-path.-chem. Analyse, Berlin, 8th edition, 1909, 571.
13. Zweifel, P., *Arch. Gynäk.*, 1904, lxxii, 1; 1905, lxxvi, 537.
14. Ten Doeschate, A., *Z. physiol. Chem.*, 1907–08, liv, 153.
15. Yoshikawa, J., *Z. physiol. Chem.*, 1913, lxxxvii, 382.
16. Gürber, A., and Grünbaum, D., *Münch. med. Woch.*, 1904, li, 377.
17. Grünbaum, *Verhandl. phys. med. Ges. Würzburg*, xxxvii, No. 3, 161.
18. Ludwig, H., *Zentr. Gynäk.*, 1895, xix, 281.
19. Wiechowski, W., *Arch. exp. Path. u. Pharmakol.*, 1909, lx, 194.
20. Wiechowski, *Beitr. chem. Physiol. u. Path.*, 1908, xi, 109.
21. Eppinger, H., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 495.
22. Cf. Salkowski in Hoppe-Seyler, Handb. physiol.-path.-chem. Analyse, Berlin, 8th edition, 1909, 153.
23. Ignatowski, A., *Z. physiol. Chem.*, 1904, xlii, 371.
24. Koettnitz, A., *Deutsch. med. Woch.*, 1889, xv, 900, 927, 949.
25. Weyl, *Arch. Anat., Physiol., u. wissensch. Med.*, 1876, 543.
26. Hohlweg, H., and Meyer, H., *Beitr. chem. Physiol. u. Path.*, 1908, xi, 381.
27. Ahlfeld, F., *Z. Geburtsh. u. Gynäk.*, 1888, xiv, 405.
28. Beale, *Monatschr. Geburtsh. u. Frauenkr.*, 1860, xvi, 157.
29. Siewert, *Z. Ges. Naturwiss.*, 1863, xxi, 146.
30. Winckel, F., Klin. Beobachtungen Path. Geburt., Rostock, 1869, 245.

31. Gusserow, A. L. S., *Arch. Gynäk.*, 1871–72, iii, 24.
32. Schöndorff, B., *Arch. ges. Physiol.*, 1899, lxxiv, 357; abstracted in *Jahresb. Fortsch. Tierchem.*, 1900, xxix, 201.
33. Brugsch, T., and Schittenhelm, A., Lehrb. klin. Untersuchungsmethoden. Berlin and Vienna, 2nd edition, 1914, ii, pt. 2, 668.
34. Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xv, 487.
35. Schroeder in Hoppe-Seyler, Handb. physiol.-path.-chem. Analyse, Berlin, 8th edition, 1909, 664.
36. Gönner, A., in von Winckel, Hand. Geburtsh., Wiesbaden, 1903, i, 320.
37. Salkowski, E., *Z. physiol. Chem.*, 1886, x, 113.

UREA FORMATION BY THE PLACENTA.

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INTRODUCTION.

The formation of urea is apparently a function of all tissues. A great deal of investigation has been carried on in an attempt to determine whether or not any particular tissue or groups of tissues possess this ability in demonstrable form, with the result that no specific urea-forming power has been found for any. The careful work of Hoagland and Mansfield[1] on muscle tissue was not productive of results favorable to the view that the formation of urea is a special property of this tissue. Nor could Fiske and Sumner[2] localize its production in liver or muscle. It would, on the surface, seem useless to attempt any study of this question with the placenta, were it not for the fact that the various methods used have produced conflicting results, and on account of the unique position of the placenta in the metabolic interchange between mother and fetus. Since the synthetic power of the placenta in so far as glycogen is concerned has been indicated by Driessen[3] and others, there is no reason to suppose that in common with other tissues it cannot form urea. As with other tissues, on autolysis leucine and tyrosine are formed by the placenta,[4] but in the literature on the placenta no mention is made of the occurrence of urea.

In view of these facts and in order to make an attempt to determine whether the placenta forms urea in determinable amounts, experiments were conducted to this end, utilizing the procedure

[1] Hoagland, R., and Mansfield, C. M., *J. Biol. Chem.*, 1917, xxxi, 487.
[2] Fiske, C. H., and Sumner, J. B., *J. Biol. Chem.*, 1914, xviii, 285.
[3] Driessen, L. F., *Arch. Gynäk.*, 1907, lxxxii, 278.
[4] Mathes, P., *Centr. Gynäk.*, 1901, xxv, 1385.

hereinafter described, which was arrived at after various unsuccessful experiments along the lines of other workers.

The following is a brief report of one phase of the work. The original idea was to make an exhaustive study of urea formation in placentas from normal and toxemic pregnancies, with a view not only to ascertain the normal course of the processes, but also to determine if possible the seat of the distressing toxemias often accompanying pregnancy. Unfortunately the war has interrupted this line of work and has prohibited a more complete survey and discussion of the problems in this paper.

Procedure.

The fresh placentas were prepared for analysis and the ammonia and urea determined as described in a previous publication.[5] Masses of the pulped tissue were then put into weighed bottles and the amounts of tissue ascertained by the increments in weight of containers and contents, usually about 150 gm. An equal weight of distilled water containing 5 cc. of a mixture of equal parts of chloroform and toluene was then added and the whole well mixed by shaking. Bits of pulp adhering to stoppers and sides of bottles were washed down with a few cc. of the diluent kept out for the purpose. The containers and contents were then put in the cold room or left in the laboratory as the occasion demanded.

Each day, after shaking the suspended pulp, the contents of the bottles were thrown onto a large funnel containing a small plug of cotton, and 10 cc. portions of the resulting filtrate were used for the analyses. The residuum in the funnel was then carefully returned to the bottles together with the washings, account being kept of the weights of added and removed fluid. In this way the removal of a fair proportion of the products of autolysis allowed the processes to continue unhindered by their accumulation.

All calculations were made to a basis of 100 gm. of fresh tissue. The results of the analyses, together with the per cent change from the initial quantities found, are recorded in Tables I to IV.

[5] Hammett, F. S., *J. Biol. Chem.*, 1918, xxxiii, 381.

TABLE I.

Urea Formation at 4°C. in Placentas from Normal Pregnancies.

Case No.	39		40		41		42		47		48		49		24		36		37	
Day.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.
	mg.	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
1	4.7	—	9.1	—	8.3	—	6.0	—	11.1	—	7.4	—	9.2	—	4.8	—	8.3	—	6.9	—
2	9.2	96	7.7	−15	10.9	31	6.3	−4	11.6	4	6.0	−19	7.8	−15	5.6	17	8.3	0	6.8	−1
3	—	—	8.8	−3	9.9	19	7.0	6	—	—	7.5	2	—	—	5.3	11	8.1	−2	6.8	−1
4	6.0	28	7.7	−15	—	—	7.7	17	12.1	8	7.6	3	9.3	1	5.7	19	8.1	−2	7.3	5
5	9.6	104	9.0	−1	12.5	50	8.1	23	12.2	10	7.9	7	10.6	16	5.8	20	—	—	—	—
6	10.3	118	9.2	1	12.6	52	—	—	12.5	13	—	—	10.2	11	—	—	8.6	4	6.2	−10
7	9.6	104	9.9	9	12.9	55	8.8	34	13.1	18	7.6	3	11.5	25	7.9	66	9.0	8	6.0	−13

TABLE II.

Urea Formation at 16°C. in Placentas from Normal Pregnancies.

Case No.	39		40		41		42		47		48		49		50		51		52	
Day.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.
	mg.	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
1	4.7	—	9.1	—	8.3	—	6.6	—	11.1	—	7.4	—	9.2	—	8.5	—	7.7	—	12.9	—
2	10.9	132	13.4	47	11.3	36	7.6	15	14.5	31	8.4	13	10.0	8	12.9	52	9.1	18	13.4	4
3	—	—	11.9	30	13.3	61	10.1	53	—	—	9.2	24	—	—	13.2	55	11.2	45	17.7	37
4	10.5	122	19.8	118	—	—	11.2	70	16.4	48	10.5	42	12.6	37	13.3	57	11.9	55	17.0	31
5	13.0	176	22.8	150	14.7	77	13.1	99	17.3	56	11.8	59	13.9	51	17.1	100	14.4	87	18.9	47
6	11.9	154	24.2	168	15.8	91	—	—	18.6	68	—	—	15.2	65	18.0	111	14.0	81	19.7	53
7	14.1	201	26.1	187	15.4	86	13.1	99	17.9	61	11.9	61	15.2	65	19.0	122	13.9	80	21.0	62

TABLE III.

Urea Formation at 4°C. in Placentas from Toxemic Pregnancies.

Case No.	46		53		25		26		27		29		30		31		32		33	
Day.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.
	mg.	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
1	24.4	—	10.2	—	5.8	—	4.4	—	15.7	—	5.6	—	4.9	—	2.5	—	14.6	—	5.3	—
2	—	—	11.6	14	6.2	7	6.4	45	—	—	8.4	50	5.3	8	7.3	192	13.9	−5	—	—
3	28.4	16	12.1	19	6.9	19	8.5	94	15.0	−4	10.9	94	8.0	65	8.1	223	14.9	2	6.6	25
4	34.3	41	12.5	22	—	—	—	—	16.9	8	12.6	126	9.2	88	9.5	279	14.1	−4	8.3	56
5	34.8	43	—	—	7.1	22	9.0	104	13.1	−17	—	—	—	—	—	—	—	—	10.1	91
6	34.3	41	12.4	21	8.3	42	9.0	104	17.2	11	12.9	131	8.7	78	7.7	207	15.6	7	11.0	104
7	36.3	49	12.6	24	7.8	34	5.2	18	19.5	24	13.2	136	9.1	86	8.2	228	17.2	18	11.8	122

TABLE IV.

Urea Formation at 16°C. in Placentas from Toxemic Pregnancies.

Case No.	46		53		38		43		44		45		54		55		56	
Day.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.	Urea N.	Increase.
	mg.	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
1	24.4	—	10.2	—	9.0	—	4.7	—	8.2	—	7.2	—	7.6	—	15.1	—	10.0	—
2	—	—	13.1	28	12.4	38	4.0	−15	13.2	61	9.7	35	9.9	30	15.1	0	10.0	0
3	29.0	19	14.4	42	—	—	8.6	83	15.3	87	—	—	12.8	69	14.9	−2	14.7	47
4	35.5	45	15.2	49	15.7	75	—	—	—	—	12.7	76	13.4	76	15.7	4	14.8	48
5	37.3	53	—	—	10.5	17	12.8	173	20.5	151	13.9	93	14.7	94	—	—	—	—
6	38.1	56	16.4	61	12.9	43	12.7	170	18.8	128	14.0	94	—	—	14.7	−3	15.9	59
7	38.8	59	16.9	66	12.7	41	12.7	170	19.3	135	15.1	110	17.2	126	14.4	−5	16.4	64

Results.

An inspection of Tables I to IV at once reveals the fact that urea, as determined by the soy bean urease, increases in amount from day to day in watery suspension of pulped placenta tissue kept in glass bottles and in the presence of chloroform and toluene.

As would be expected, an increase in the temperature at which the tissues were kept resulted in an increase in the amounts of urea formed in a given period. This is plainly shown by a comparison at the two temperatures of Cases 39, 40, 41, 42, 47, 48, and 49 in Tables I and II, of placentas from normal pregnancies; and a like comparison in Cases 46 and 53, Tables III and IV, of placentas from toxemic pregnancies.

An insufficient number of correlated cases from toxemic pregnancies prevents any conclusion as to the relativity of the action involved.

DISCUSSION.

It is evident that the placenta can form urea under certain circumstances. The processes leading up to this product are in question. It may be simply a splitting of the urea residue from arginase, or it may be a heightening to determinability of the usual processes taking place in extrauterine life, whose course from deaminization, through ammonium carbamate, to urea, is fairly well established.

Whatever the source of the urea found to be formed by placenta tissue, these results give evidence that this organ as well as being excretory in function, is also actively concerned in the preparation of the nitrogenous by-products of the fetus for excretion.

It is our present idea that the fetus is only faintly able if at all, to perform for, of, and by itself the functions of preparing its nourishment in assimilable form, or of breaking down its by-products into compounds ready for excretion; and that the placenta is interposed between mother and fetus to remove as far as possible these extra burdens from the maternal organism. These questions can obviously be settled only by continuation of the study of the biochemistry of placenta and fetal tissues.

CONCLUSION.

Urea formation is one of the properties of the placenta that can be conclusively demonstrated, although the processes leading up to its production can only be surmised. This finding lends further importance to the placenta as an intermediary organ between mother and fetus for mutual protection, as well as for the carrying on of processes undeveloped by the fetus, yet necessary for its continuance.

THE MAXIMUM PRODUCTION OF GLUTAMINE BY THE HUMAN BODY AS MEASURED BY THE OUTPUT OF PHENYLACETYLGLUTAMINE.

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(Received for publication, November 12, 1918.)

Phenylacetic acid when fed to animals such as horses (1), dogs, rabbits (2), and sheep (3), is excreted in the urine as phenaceturic acid. This combination of phenylacetic acid with glycocoll, however, does not take place in the human body. In man the phenylacetic acid combines with glutamine and is excreted in the urine as phenylacetylglutamine (4). E. Salkowski and H. Salkowski (2) find after feeding phenylacetic acid to rabbits an increase of almost 75 per cent in the total SO_3 output, but at the same time a decrease in the amount of "combined SO_3." The increase in the amount of inorganic sulfates in the urine they attribute to an increased catabolism of protein material necessary for the preparation of sufficient amount of glycocoll to detoxicate the phenylacetic acid. The decrease in ethereal sulfates they believed to be due to the action of phenylacetic acid as a disinfecting agent with a subsequent decrease in the amount of intestinal putrefaction.

As glutamine (5) rather than glutamic acid seems to be the primary amino-acid constituent of protein, we thought it important to determine the maximum amount of this acid which the human body is able to furnish for the detoxication of phenylacetic acid. We wished also to know whether the ingestion of phenylacetic acid would be followed by increased sulfur metabolism in the human body as reported by previous investigators (2) in animals. Lastly, we wished to determine the effect of the acid in the course of intestinal putrefaction as measured by the excretion of urinary indican, as well as by the amount of ethereal sulfates excreted in the urine.

EXPERIMENTAL.

The subject was a man weighing 60.2 kilos. He was maintained throughout the entire experimental period on a uniform diet. The experiment covered a period of 24 days. On the 5th day of this period 2.5 gm. of phenylacetic acid were ingested, on the 7th day 5 gm., on the 10th day 7.5 gm., on the 13th day 10 gm., and on the 21st day 15 gm. The acid in each case was ingested as a solution of the sodium salt. The entire amount of the solution was taken at once, covering a period of not more than 3 to 5 minutes. The urine was collected in 24 hour periods, beginning at the time of the ingestion of the acid. The urine volume was first measured, and sufficient amount taken for the indican and sulfate determinations on that day and the day following the acid ingestion; the remaining urine volume was measured, exactly neutralized with sodium carbonate, and evaporated on the water bath to a thick syrup. The determination of the indican was made in duplicate upon each urine sample by the method of Ellinger (6). Each cc. of the Wang solution (7) employed in the titration was found upon standardization to be equivalent to 0.300 mg. of indigo,[1] or 0.576 mg. of indican. The ethereal sulfates and total sulfates in the urine were determined by the method of Folin (8). To determine the quantity of phenylacetylglutamine, the evaporated urine was made acid with phosphoric acid (Congo red as indicator) and extracted in a continuous extracting apparatus with absolute ethyl acetate. The amount of ethyl acetate used during each extraction was about 300 cc., and the time of extraction varied between 1 and 2 hours. The extracting was continued until the ethyl acetate was found on evaporation to contain no more phenylacetylglutamine. To obtain the phenylacetylglutamine the ethyl acetate extracts were placed on ice for 24 hours. At the end of this time, the white flaky crystals of the substance appeared on the sides and bottom of the flask. These crystals were scraped loose and filtered off, the mother liquor was evaporated to one-half the volume, and again placed in the ice box for 24 hours. This process was repeated until all the compound was crystallized out of the solution. The

[1] The indigo used in standardizing the Wang solution was furnished by Professor P. B. Hawk of Jefferson Medical College.

phenylacetylglutamine from the different extractions was united and recrystallized from absolute ethyl acetate, dried, and weighed. In each extract there occurred about an equal amount of phenylacetylglutamine and phenylacetylglutamine urea. In order to split off the urea and convert the urea compound into phenylacetylglutamine, a water solution of the urea compound was made slightly alkaline with barium hydroxide, the solution treated with carbon dioxide to remove the excess of barium, filtered, and the filtrate evaporated to dryness at a low temperature on the water bath. The residue, consisting of the barium salt of phenylacetylglutamine and urea, was extracted several times with hot absolute alcohol to remove the urea. The insoluble barium salt was separated from the urea by filtration. The barium salt of the phenylacetylglutamine was dissolved in a small amount of water, made acid with phosphoric acid, the barium sulfate filtered off, and the phenylacetylglutamine extracted from the concentrated solution with ethyl acetate.

DISCUSSION.

The results of the experiment are summed up in Table I.

On account of the toxicity of the phenylacetic acid, it was impossible for the subject to ingest more than 15 gm. of the acid. Even 5 gm. caused thirst, a slight feeling of dizziness, and nausea. The 10 gm. dose seemed to produce no exaggeration of these symptoms, but after ingestion of 15 gm. the subject noticed marked signs of poisoning, not unlike those following the ingestion of large quantities of alcohol. The daily urine volume which generally measured 800 to 900 cc. rose to at least 1,000 cc., and in one instance to 1,300 cc. after the ingestion of more than 5 gm. of phenylacetic acid. The increase in sulfur metabolism noted by Salkowski and Salkowski (2) after feeding the same acid to rabbits is not borne out in this experiment.[2] On the 13th day of the experiment after an ingestion of 10 gm. of the acid, there was a rise in the total SO_4 output from 1.6862 gm. on the previous day to 2.5107 gm. This, although a marked rise, is even lower than the amount excreted the 3rd day before the ingestion of any of the acid.

[2] The effect of phenylacetylglutamine production on nitrogen metabolism is being studied and will be reported later.

TABLE I.

Day of experiment.	24 hr. urine, volume.	Phenylacetic acid ingested.	Phenylacetylglutamine recovered from urine.	Phenylacetic acid conjugated with glutamine.	Phenylacetic acid recovered from urine as phenylacetylglutamine.	Glutamine extracted from urine as phenylacetylglutamine.	Total SO_4 excreted in urine during 24 hrs.	Inorganic SO_4 excreted in urine during 24 hrs.	Ethereal SO_4 excreted in urine during 24 hrs.	Indican excreted in urine during 24 hrs.	Relation of ethereal SO_4 to total SO_4 for 24 hr. period.	Relation of indican SO_4 to ethereal SO_4.
	cc.	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*		
1	860						1.8049	1.5905	214.4	23.82	1: 8.41	1: 19.96
2	850						1.8467	1.6843	162.4	27.26	1: 11.37	1: 13.21
3	960						2.6013	2.3340	267.3	26.44	1: 9.73	1: 22.42
4	920						2.5340	2.3518	182.2	22.86	1: 13.41	1: 17.67
5	900	2.5	2.02	1.1332	52.88	1.1070	2.1040	1.9683	135.7	12.90	1: 15.48	1: 23.33
6	1,010						2.7792	2.5702	209.0	28.16	1: 13.31	1: 16.46
7	1,100	5.0	6.16	3.3820	67.67	3.3264	2.7491	2.5848	164.3	00.00	1: 16.73	1: 00.00
8	970						1.9050	1.6972	207.8	00.00	1: 9.16	1: 00.00
9	820						2.3440	2.1473	196.7	12.66	1: 11.91	1: 34.44
10	1,070	7.5	7.23	3.7241	49.65	3.6873	2.4844	2.3516	132.8	14.28	1: 14.86	1: 20.63
11	820						2.0066	1.9471	159.5	9.76	1: 12.54	1: 36.24
12	785						1.6862	1.5314	154.8	5.39	1: 10.89	1: 63.64
13	950	10.0	10.07	5.1870	51.87	5.1357	2.5107	2.3237	167.0	31.89	1: 15.03	1: 12.00
14	840						1.6403	1.4835	156.8	27.32	1: 10.46	1: 12.76
15	700						1.8446	1.6903	154.3	28.11	1: 11.95	1: 12.18
16	900						1.7648	1.6272	137.6	29.02	1: 12.82	1: 10.53
17	1,200						1.6680	1.4850	183.0	30.24	1: 9.11	1: 13.42
18	690						1.5998	1.4688	131.0	19.57	1: 12.21	1: 14.84
19	815						1.7077	1.5573	150.4	22.61	1: 11.35	1: 14.75
20	826						2.3319	2.0873	244.6	26.59	1: 9.53	1: 20.40
21	1,305	15.0	14.75	7.5977	50.65	7.5225	2.0398	1.9034	105.5	22.39	1: 19.31	1: 10.46
22	860						2.2621	2.0654	196.7	40.87	1: 11.49	1: 10.65
23	780						1.9707	1.8000	164.9	6.27	1: 11.95	1: 58.30
24	720						1.8294	1.6830	146.4	8.70	1: 12.50	1: 37.32

On the 10th day, after receiving 7.5 gm. of the acid, there was an increase in total SO_4 of 0.1404 gm. or 5.95 per cent over the previous day. In all other cases there is even a decrease in the total amount of SO_4 excreted in the urine. There is no marked decrease in the amount of ethereal sulfates after the ingestion of the acid except on the 21st day of the experiment, when the amount fell to less than half the amount of the previous day and was approximately one-half the amount excreted on the succeeding day. There was also a relative decrease in ethereal sulfates in every case as is shown by the proportion of total SO_4 to ethereal SO_4.

The influence of the phenylacetic acid on putrefaction in the intestine is best measured by the indican (9) output in the urine, as the amount in each case is affected by the ingestion of the acid. The influence of the phenylacetic acid on the indican excretion is most marked on the 1st or 2nd day after the ingestion of the acid. After a 5 gm. dose of the acid, the indican entirely disappeared for the same day and the day following, while on the 13th day 10 gm. of the acid caused only a slight decrease in the amount of indican excreted. After the ingestion of 15 gm., there is a very slight drop in the 24 hour indican value followed the next day by a marked increase. The decided lowering of the indican content of the urine first appeared on the 2nd and 3rd days after acid was taken. The ratio of indican SO_4 to total ethereal SO_4 shows that the production of indican was retarded to a much greater extent than the other compounds constituting the remainder of the ethereal sulfates.

In every case the amount of glutamine furnished by the body for the detoxication of the phenylacetic acid was approximately one-half the necessary amount. Regardless of the size of the dose of acid, only about 50 per cent of the acid appeared in the urine as phenylacetylglutamine.

After a dose of 2.5 gm., 2.02 gm. of the compound, corresponding to 52.88 per cent of the acid ingested, were isolated from the urine. After ingesting 15 gm. of the acid, 14.75 gm. of phenylacetylglutamine were recovered from the urine. In this case 50.65 per cent of the acid ingested was recovered. An exception was found after a 5 gm. dose, when the amount of acid regained amounted to 67.67 per cent with a collection of 3.3820 gm. of the

compound. The amount of glutamine furnished in each case was proportional to the amount of phenylacetic acid ingested regardless of the size of the dose. The largest quantity of glutamine isolated from the urine in combination with the phenylacetic acid was 7.5225 gm. after a dose of 15 gm. of the acid. It is probable that more of the phenylacetylglutamine would have appeared in the urine after each dose of the acid, had the acid been ingested at regular intervals covering a period of 10 or 12 hours. There may have been a reversible reaction at work thus decreasing the amount of the phenylacetic acid compound in the urine, or the remainder of the acid yet unaccounted for may have been detoxicated and eliminated through the urine as an entirely different compound.

CONCLUSION.

1. No marked increase in sulfur metabolism followed the ingestion of phenylacetic acid by man, as is shown by the very slight increase in the total SO_4 content of the urine. This does not agree with the findings of the experiment on rabbits by Salkowski and Salkowski.

2. Intestinal putrefaction is decreased after ingesting phenylacetic acid. The amount of urinary indican was decreased as well as the total ethereal sulfates.

3. About 50 per cent of the phenylacetic acid ingested was detoxicated in each case by uniting with glutamine. The largest amount of glutamine excreted was 7.5225 gm. after a dose of 15 gm. of phenylacetic acid. The amount of phenylacetylglutamine isolated from the urine after the 15 gm. dose of the acid was 14.75 gm.

BIBLIOGRAPHY.

1. Salkowski, E., *Ber. chem. Ges.*, 1884, xvii, 3010; *Z. physiol. Chem.*, 1885, ix, 229.
2. Salkowski, E., and Salkowski, H., *Ber. chem. Ges.*, 1879, xii, 653; *Z. physiol. Chem.*, 1882–83, vii, 161.
3. Vasiliu, *Mitt. Landw. Inst. Univ. Breslau*, 1909, iv, 703.
4. Thierfelder, H., and Sherwin, C. P., *Ber. chem. Ges.*, 1914, xlvii, 2630; *Z. physiol. Chem.*, 1915, xciv, 1.

5. Sherwin, C. P., Inaugural dissertation, Tübingen, 1915. Fischer, E., and Koenigs, E., *Ber. chem. Ges.*, 1904, xxxvii, 4585. Osborne, T. B., and Gilbert, R. D., *Am. J. Physiol.*, 1905–06, xv, 333. Osborne, T. B., Leavenworth, C. S., and Brautlecht, C. A., *Am. J. Physiol.*, 1908–09, xxiii, 184. Abderhalden, E., Lehrb. physiol. Chem., Berlin, 1915, 3rd edition, ii, 1169.
6. Ellinger, A., *Z. physiol. Chem.*, 1903, xxxviii, 192.
7. Wang, E., *Z. physiol. Chem.*, 1898, xxv, 409.
8. Folin, O., *Am. J. Physiol.*, 1905, xiii, 52.
9. Folin, *Am. J. Physiol.*, 1905, xiii, 99.

BEHAVIOR OF SUDAN III IN THE ANIMAL ORGANISM.

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The fat-soluble dye Sudan III, since its first preparation by Nietzki[1] in 1880, has been used extensively in the study of problems directly and indirectly associated with vital staining and the metabolism of fat. The work of Daddi, Gage, Riddle, Mendel, Whitehead, and many others, including that recently published by Hatai,[2] has shown how fat can be traced in the body by means of oil-soluble dyes.

In their paper on the behavior of fat-soluble dyes in the animal organism, Mendel and Daniels[3] discussed the toxicity, absorption, transport, and excretion of this dye. They stated that it is apparently non-toxic, inasmuch as animals fed over long periods of time showed no deleterious results. When fed in solution in fat it may enter the organism from the alimentary tract through the lymphatics or by the portal circulation dissolved in reabsorbed bile, a cycle becoming established between the intestine, bile, and blood. The dye is eliminated in the feces alone, except when an alimentary lipuria arises; then it may occur in the urine. Moreover, it is deposited in the adipose tissues and bone marrow, stained fat being no less available to the organism than unstained.

These findings were later discussed by Salant and Bengis[4] in their physiological and pharmacological studies of fat-soluble dyes. Using exceedingly large doses, they found that Sudan III was not pronouncedly toxic. Administered intraperitoneally, subcutaneously, intravenously, or by mouth the dye was reported

[1] Nietzki, R., *Ber. chem. Ges.*, 1880, xiii, 800.

[2] Hatai, S., *J. Exp. Zool.*, 1918, xxvi, 101.

[3] Mendel, L. B., and Daniels, A. L., *J. Biol. Chem.*, 1912–13, xiii, 71.

[4] Salant, W., and Bengis, R., *J. Biol. Chem.*, 1916, xxvii, 403.

to be excreted by the kidney, sometimes found in the bile, and deposited in the adipose tissue only. When observed a few hours after experiment, the central nervous system sometimes showed faint traces of color. Salant and Bengis contended that the absence of dye in the urine as reported by Mendel and Daniels[3] was due to the abnormality of the animals, because only in the case of diseased animals did Salant and Bengis find absence of dye in the urine. This outcome they ascribe to decreased permeability of the kidneys.

The recent work of Hatai[2] on the several effects of feeding Sudan III to rats may well be subjected to criticism and maybe to revision in the light of the results reported below. He found that the feeding of Sudan III to young rats retarded the growth of the thymus, testes, and ovaries. Postmortem examination showed no trace of dye in the brain, spleen, or heart; but its presence was noted in the liver, pancreas, and kidney.

The original purpose of the present experiments was to reinvestigate the seemingly contradictory statements with regard to the path of elimination of the fat-soluble dye Sudan III, and particularly the contention of Salant and Bengis regarding the excretion of Sudan III by the kidneys. The products of various manufacturers were used and gave varying results, which led to interesting observations on the doubtful purity of preparations of Sudan III obtainable on the market at present. Experiments made with a purified product, on the other hand, gave definite results, thus showing the need for careful examination of all preparations of Sudan III used in scientific work to guarantee its purity before drawing conclusions supposedly based upon its properties as a pure, neutral fat-soluble dye.

Properties of Sudan III.

There are many references in the literature to the preparation and properties of Sudan III, which may be briefly summarized as follows:

Sudan III, $C_{22}H_{16}N_4O$. Synonyms.[5]

Benzeneazobenzeneazo-β-naphthol, $C_6H_5N=N-C_6H_4-N=N-C_{10}H_6OH$. Tetrazobenzol-β-naphthol, Oil Red O, Cerasinrot, Scharlach R. Fettponceau, Motirot 2R, etc. (Some are the methylated compounds.)

[5] Merck's 1907 Index, New York, 3rd edition, 1907, 429.

It is a brick-red powder, insoluble in water, alkali, or dilute acid; slightly soluble in alcohol; very readily soluble in hot glacial acetic acid, chloroform, ether, paraffin, benzene, oils and fats, fatty acids, and all lipoid solvents. It may be crystallized out of glacial acetic acid in sheeny metal green-brown plates, melting at 195°C.

When treated with strong sulfuric acid it gives a green color. It splits into amino-naphthol, aniline, and *p*-phenylenediamine when treated with tin and hydrochloric acid. The sodium salt of the sulfonated compound is known as B Scharlach, a much used water-soluble dye.

The peculiar solubilities of Sudan III have facilitated the study of fat digestion and absorption through the intestinal walls. It is even more soluble in bile than in fat, but the question of its solubility in soaps or glycerol does not seem clear. Pflüger[6] states that the pure dye is freely soluble in bile and dissolves in both soap and glycerol giving a deep red solution. Mendel,[7] while reaffirming its solubility in bile even in preference to oil, states on the contrary that it is insoluble in either glycerol or soap solution.

Bearing in mind the above properties we now turn to the examination of products on the market which have been in common use. The samples taken were those put out by (1) Kahlbaum, (2) Berlin Anilin Fabrik, (3) Grübler, (4) A. H. Thomas Co.

Specimen.	Melting point.	Color.	Remarks.
	°C.		
1	130	Brick-red powder.	Very readily soluble in oil.
2	120	Dark venous-red.	Solution in oil dark colored.
3	135	Dark brick-red, lumps showing lighter material inside.	Very readily soluble in oil.
4	120	Orange-red powder.	Solution in oil of deep orange color, more or less soluble in dilute alkalies and acids.
5	195	Green-brown sheeny plates.	Purified product.

[6] Pflüger, E., *Arch. ges. Physiol.*, 1900, lxxxi, 375.
[7] Mendel, L. B., *Am. J. Physiol.*, 1909, xxiv, 493.

These varying properties show unmistakably the presence of impurities in the commercial products.

Purification of Sudan III.

The dye may be separated from its impurities by first dissolving in alcohol or glacial acetic acid, and subsequently precipitating in water to remove all water-soluble substances; the repetition of this procedure results in a fine brick-red powder of low melting point similar to that of the commercial article. It should then be recrystallized out of hot glacial acetic acid three or more times, the purity of the fine sheeny flocculent crystals thus obtained being judged by the melting point. Very impure products need to be dissolved in a lipoid solvent and shaken out with dilute aqueous alkali and acid alternately until the aqueous solvent ceases to take up any further color.

The solubility of Sudan III in oil has been the subject of long discussion. Michaelis[8] considered that it was readily soluble in oil, but does not state to what degree; on the other hand Mann[9] considered the solution to depend on a chemical and not a physical process. Mann concludes from Michaelis' own work that the union between Sudan III and oleic acid is a chemical one depending on the oxidation of the unsaturated fatty compound, analogous to the action of osmium tetraoxide, the only difference being that azo dyes form additive compounds with the fat without changing their color, while osmium compounds are readily decomposed.

The chemistry of Sudan III has been worked out in the elaborate researches of Michaelis. He showed that its staining power was dependent on the simple azo group "benzene azobetanaphthol," and varied according to the nature of the rest of the molecule.

Bell[10] in his experiments on the interstitial granules of striated muscle secured his best results with Herxheimer's stain, and relative to the experiments here reported it should be noted that he states, "very variable results were obtained," indicating that the

[8] Michaelis, L., *Virchows Arch. path. Anat.*, 1901, clxiv, 263.

[9] Mann, G., Physiological histology, Oxford, 1902, 306.

[10] Bell, E. T., *Internat. Monatschr. Anat. u. Physiol.*, 1911, xxviii, 297.

quality of the dye varied, although "only Grübler's dyes have been used." The methyl and ethyl compounds of benzeneazonaphthol have a greater staining power—especially when no salt-forming groups are present. The "indifferent" character of Sudan III makes it a peculiarly efficient fat-soluble dye. It has great ability to unite with fat cells in both an acid and alkaline medium as compared with other dyes. This is shown by Robertson[11] in his work on tissue staining.

The solubility of Sudan III in oil has an important bearing on the relative availability of stained and unstained fat in the animal body. The assimilation of a solution of the dye would be expected to follow a different path from that of the assimilation of an oil compound, the latter possibly rendering the fat unavailable for use in the organism.

Unlike Scharlach R, Sudan III dissolved in alcohol and mixed with five to six volumes of water does not form a colloidal solution, and apparently does not show the same adsorption phenomena as do some other indifferent dyes. This property should be considered in the interpretation of the supposed urinary excretion of Sudan III.

Sudan III is easily detectable in the body fluids. Simple solution of the dye in alcohol gives a distinct pink color up to one part in ten million; likewise in any of the lipoid solvents when it is present in traces (one part in a million). Gasoline, in preference to ether or toluene, was found to be the cheapest and best solvent. The writer did not find Salant's[4] wool method satisfactory for dark colored urines. The wool adsorbs so much of the urinary pigment that it is almost impossible to tell whether there is any dye present. Sudan III is best extracted from lymph by a mixture of one volume of alcohol and two volumes of ether, a quantitative yield being obtained by shaking the mixture and standing it aside for 24 hours.

Absorption and Transport.

Mottram[12] showed clearly that animals possess varying degrees of ability to absorb and use fat; therefore one might expect

[11] Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1.
[12] Mottram, V. H., *J. Physiol.*, 1915–16, l, 380.

to find some variation in the results obtained by workers using different animals. Mendel worked chiefly with dog, cats, and rats; Salant used rabbits, the normal diet of which includes very little fat. In one of the following experiments the writer injected intraperitoneally an aqueous suspension of the dye into a rabbit and observed no excretion either in the urine or feces. The animal when killed showed a pink omentum, unstained below the surface, with scattered lumps of dye embedded in it. Intraperitoneal injection of the dye dissolved in oil gave good absorption and excretion, the dye appearing in the feces in 1 to 5 days. Rats injected intraperitoneally showed little absorption of the stained oil. The rats were killed after 2 or 3 weeks and there was found still present in the peritoneum a considerable amount of stained oil. Mendel[3] has shown beyond question that when Sudan III is fed by mouth it passes readily into the lymphatics and thence into the blood stream; later it is eliminated with the bile into the alimentary tract.

There are other factors which influence the nature and rate of absorption of fat and therefore of stained fat. Various adjuncts have been used to assist in emulsifying the oil administered. Mendel used lecithin, a substance which Loevenhart and Souder[13] showed to be an accelerator to the digestive action of pancreatic juice on oils. Salant[4] used emulsions containing sodium carbonate, which would greatly decrease the solubility of the dye in the oil and so probably retard its absorption. Salant also used suspensions in gelatin, which might or might not be absorbed depending on the condition of the animal, such a preparation being comparable to a watery suspension.

In view of the above facts regarding the doubtful absolute purity of some of the preparations of Sudan III heretofore used, the question of the absorption of fat through the intestinal mucosa might well be brought forward again. As the dye has been relied upon as an exact index of the paths of absorption and transport of fat, any small amount of a foreign coloring matter present may give very misleading results, from which erroneous conclusions may have been drawn.

[13] Loevenhart, A. S., and Souder, C. G., *J. Biol. Chem.*, 1906–07, ii, 415.

Excretion.

The work of Abel and Rowntree[14] on the excretion of dyes leads one to expect Sudan III, on account of its indifferent character, to be excreted in the feces. This was found to be the case by Mendel[3] and was confirmed in the present work on dogs, all of which were tested by the phenolsulfonephthalein test and found normal in respect to kidney function. Salant claimed that the dye was excreted in the urine, but obtained varying results from animals with deranged kidney mechanism, his experiments being conducted with rabbits to which very large doses of dye were given. The writer repeated this work with rabbits and found variation according to the purity of the dye, the size of the dose, and the kind of diet. In all cases *when the pure dye was fed, even in large amount, no dye was excreted in the urine,* soft deep red stools being obtained in 2 to 4 hours.

Small doses of the unpurified product given intraperitoneally did not appear in the urine. It was impossible to give to rabbits such large amounts intraperitoneally as by mouth. Large doses of the crude drug—as much as 2 gm. at one time—were administered with the aid of a stomach tube to full grown rabbits. During one or more days following, dark reddish brown urines were obtained. The dark red-brown pigment was not Sudan III. It was thought to be probably a foreign pigment present in the original impure dye, soluble in the body fluids, and excretable by the kidney in some form or other. This should not be interpreted as characteristic of the excretion of Sudan III. Pure Sudan III so administered to rabbits did not give pink urine; and the same negative outcome was observed when dogs were given moderate doses of the impure products with their daily food. The colored urines were only obtained when abnormally large doses of the crude dye with its impurities were administered to rabbits. The urines on standing deposited the pigment as a dark brown precipitate. The amount of the pigment seemed much increased when the rabbits were given exclusively an oat diet. Such a diet with its resulting acidosis changes the nature of the renal excre-

[14] Abel, J. J., and Rowntree, L. G., *J. Pharm. and Exp. Therap.*, 1909–10, i, 231.

tion, and herein may lie the explanation of the appearance of the pigment impurity in the urine.

The *pure* dye is excreted in the feces only, the earliest appearance observed being after 2 hours. It is extractable by gasoline, whereby it may be estimated quantitatively.

Toxicity.

Weyl[15] some years ago announced that diazo colors in general are non-poisonous. This has been confirmed in particular with regard to Sudan III by subsequent workers. Mendel stated Sudan III to be harmless even in large doses provided the dye was pure. Salant and Bengis in their experiments observed death after 1 to 3 weeks; but they regarded it as extremely doubtful whether death was due to the dye. Hatai reported that Sudan III retarded to a considerable extent the body growth of albino rats, and caused a striking change in weight of many of the organs, especially the liver, pancreas, thymus, testes, and ovaries.

The following experiments show that the *impure* dye may be markedly toxic. When 2 gm. were given at one time to normal rabbits, in all cases death ensued in a very short time. A neuritic-like condition of the hind legs developed first, and sooner or later the animals died in convulsions. Rabbits fed on an exclusive oat diet showed paralytic symptoms very rapidly after feeding the dye. The pure dye given in normal amount did not show any of these symptoms.

A number of albino rats were used to test further the toxicity of the various products. Those fed the crude preparations became very much debilitated. The orange colored dye (No. 4) stunted the growth, and brought about a general appearance of malnutrition; and in other rats the hind legs were paralyzed on one or both sides.

Path of Absorption and Excretion of Sudan III in Animals with Normal Kidney Mechanism.

Dogs were fed moderate doses of Sudan III in fatty food, and after several hours cannulas were inserted in the thoracic and bile

[15] Weyl, T., Die Teerfarben, Berlin, 1889.

ducts to collect the lymph and the bile. Blood was taken from the femoral artery. Just before death the bladder was emptied with great care to avoid contamination, and the urine examined for the presence of the dye. All the animals were tested several hours before operation with the phenolsulfonephthalein kidney function test and were found to be normal.

The following results show that the dye administered with fat is well absorbed into the lymphatics, is transported into the blood, and excreted with the bile, and reabsorbed in it from the intestine. It is not excreted by the kidney, but always appears in the feces. Small doses of the crude products did not influence the color of the urine.

Experiment 1.—Dog, female, weight 13.5 kilos. Fed 4 gm. of Sudan III Specimen 1 during 1 week, at the same time adding plenty of lard to the diet. Very soft pink feces were excreted. On the 8th day, 3 hours after taking a heavy fat meal containing Sudan III the animal was operated upon to obtain samples of lymph and bile. Both contained a considerable amount of Sudan III. The milky lymph was intensely pink, and the dark bile, dried down with lime and extracted with ether, showed an abundance of Sudan III. The urine showed no trace of red color. At the conclusion of the operation 1 cc. of phenolsulfonephthalein was injected subcutaneously, and after 9 minutes 2 cc. of urine were obtained giving a strong positive reaction.

Experiment 2.—Dog, male, weight 14.3 kilos. 1 gm. of Sudan III Specimen 1 was added with 15 gm. of lard to the morning meal of ground biscuit. After 5 hours the animal showed signs of gastric disturbance; there was vomiting with diarrhea and it was several days before the animal appeared normal again. When fully recovered, the animal was given on 3 successive days 1 gm. of purified Sudan III with the morning meal. On the 3rd day, 1 hour after feeding, an operation was conducted to obtain lymph and bile. More than 100 cc. of a very pink lymph were obtained in 2 hours, and the bile collected was saturated with Sudan III. No dye appeared in the urine. The pure dye did not disturb the digestive system so much as the crude products.

Experiment 3.—Dog, female, weight 10.5 kilos. 0.5 gm. of pure dye was fed with 10 gm. of lard, and 2 hours later a similar amount was fed in half a pint of cream. After 4 hours the animal was operated upon as in Experiments 1 and 2. The lymph was decidedly pink, and the bile taken direct from the gall bladder when extracted with gasoline showed the presence of a considerable amount of Sudan III. There was no trace of dye in the urine. At the end of the operation 0.5 cc. of phenolsulfonephthalein solution was injected subcutaneously, and after 7 minutes it appeared in the urine showing that the kidneys were functioning well.

Experiment 4.—Dog, female, weight 12.4 kilos. Fed 1 gm. of pure dye, and after 2 days the dose was repeated with half a pint of cream. 1 hour after giving the second dose the dog was operated upon as before. The lymph flow was exceedingly small but quite pink in color. The bile gave a strong red color when shaken up with gasoline. Blood collected from the femoral artery was allowed to clot and the serum extracted with gasoline. It showed unmistakably the presence of the dye. There was no trace of dye in the urine.

Experiment 5.—Dog, male, weight 11.5 kilos. 1.5 gm. of Sudan III Specimen 1 with lard were added to the usual food. After 1½ hours an operation was conducted to obtain specimens of lymph, bile, blood, and urine. All except the urine showed the presence of Sudan III in marked amount.

Experiment 6.—Dog, female, weight 14.5 kilos. 1 gm. of Sudan III Specimen 4 was given with lard, cream, and biscuits. After 2 hours the body fluids were collected as in Experiment 5. The lymph and blood were strongly positive, but the bile was much lighter than usual containing only very faint traces of the dye. The urine, as in all of the experiments, showed no trace of Sudan III.

TABLE I.

Summary of Experiments on Dogs.

Experiment No.	Dye.	Sudan III in			
		Lymph.	Bile.	Blood.	Urine.
1	1	++++	++++	—	0
2	Pure.	++++	++++	—	0
3	"	++	++	—	0
4	"	+	++	+	0
5	1	++	++	++	0
6	4	++	+	++	0

The results with normal animals confirm the findings of Mendel and Daniels,[3] and answer clearly the criticism of Salant and Bengis.[4]

To investigate further the nature of the absorption and excretion of Sudan III, experiments similar to those of Salant and Bengis were conducted on rabbits. The protocols of the various experiments bring out especially the toxicity of the crude products in common use.

Experiment 7. Toxicity of Commercial Sudan III, Specimen 1.—Rabbit 1, female, weight 1.6 kilos. Mixed diet (oats, corn, cabbage, and carrots). 84 mg. were injected intraperitoneally with 5 cc. of oil. During the following 3 days soft, deep red feces were obtained but the urine was nor-

mal. Similarly a week later 126 mg. were given in 10 cc. of oil. 8 days later 2.72 gm. in 20 cc. of oil were given by mouth. Intensely red soft feces were excreted in 3½ hours. Subsequent diarrhea made it difficult to keep the animal clean, the belly and hind quarters becoming smeared very red. The toxic symptoms of the dye developed gradually; after a few hours there was loss of control in the hind limbs and sprawling on the front legs, and the animal died in convulsions within 24 hours. No dye appeared in the urine at any time.

Experiment 8. Effect of Administering Sudan III, Specimen 1, Recrystallized Three Times, Melting Point 130°C., with Exclusive Oat Diet.—Rabbit 2, female, weight 1.76 kilos. The animal was fed on oats for several days, when the daily excretion of urine became very small. 92 mg. of dye were injected with 10 cc. of oil intraperitoneally. In a few days there developed paralysis in the left leg with albuminuria. The sickness continued acute as long as the exclusive oat diet was given. After 25 days carrots were added to the diet, and 2 gm. of the dye were given with 20 cc. of oil. A very heavy, dark brown urine was voided for 6 days, after which time the urine returned to its normal color, and the paralysis in the leg was much improved. The animal died several weeks later. The pigment in the urine was examined for bile pigment with a negative result. It precipitated out on standing. Hydrolyzed with acid and alkali it failed to give any red color. On account of its general properties and the fact that the administration of pure Sudan III did not produce this pigment, the pigment must have originated as an impurity in the commercial specimen of the dye.

Experiment 9. Toxicity of Commercial Sudan III, Specimen 3.—Rabbit 3, female, weight 1.7 kilos. Diet of oats and carrots. 2 gm. of the mixed impure residues from the recrystallization of the dye were given with oil by mouth. After 2 hours soft red feces were excreted, which in 4 hours changed to a red liquid stool. The urine collected during the following 6 days was a thick red colloid, after which time the animal appeared normal in every way. 12 days later 2 gm. of commercial Sudan III, Specimen 3, were given with oil by mouth. The animal excreted red-brown urine during several days, and gradually developed neuritic-like symptoms, the right side being first affected, and later the hind legs being completely paralyzed. At the end of the 3rd week the rabbit had a convulsion, and the following day died in convulsions.

Experiment 10. Effect of Administering Impure Sudan III, Specimen 4.—Rabbit 4, female, weight 1.84 kilos. Exclusive oat diet. 2 gm. of the dye suspended in water were given by mouth. On the following day only the feces were colored red, but a very dark brown urine was excreted for a week. After 3 weeks the animal was killed. It showed no stain either in the omentum or any of the fatty tissues of the body. The urine after standing a week deposited the pigment on the sides of the vessel. This brown material was examined as before, and was also apparently an impurity in the original dyestuff. There was degeneration of the liver. It was concluded that the dye failed to enter the circulation at all; otherwise at least the omentum would have been stained.

Experiment 11. Absorption, Distribution, and Excretion of Pure Sudan III Administered to an Animal on a Good Mixed Diet.—Rabbit 5, female, weight 2.0 kilos. Diet of oats, corn, cabbage, and carrots. 1 gm. was given by mouth with 50 cc. of oil. After 3½ hours very red feces were excreted and acute diarrhea followed, lasting several days. The urine was darker than normal, but the natural color returned in 5 days. The animal was killed a month later. The omentum, the fatty tissues of the neck and legs, and the kidney and heart fat all showed distinctly the presence of the dye, being stained pink. The nerves and spinal cord were quite white, and the bile was free from the dye. The pure dye was apparently non-toxic.

Experiment 12.—Repetition of Experiment 11. Rabbit 6, female, weight 2.1 kilos. Good mixed diet. 1.5 gm. of the purified material were given with 35 cc. of oil by mouth. There was absolutely no sign of any foreign pigment in the urine excreted during the following month. The dye was stored in the omentum and fatty tissues of the body, and excreted in the feces, as in the previous experiment.

Experiment 13. Effect of Injections of Small Doses of Pure Sudan III.—Rabbit 7, female, weight 1.72 kilos. Diet of oats, corn, and cabbage. 20 mg. of pure Sudan III were dissolved in 5 cc. of peanut oil and injected intraperitoneally. 5 days later 45 mg. in oil were injected subcutaneously, and after 13 days a water suspension of 65 mg. was injected intraperitoneally. There was no immediate appearance of the dye in either the urine or feces. The animal was killed a week later. It showed a heavily stained omentum, the inner layer of which was quite white. The particles of dye from the injection of the watery suspension appeared just scattered over the fatty surface, and were evidently unabsorbed.

Experiments were conducted further with white rats. The toxic effects of the crude impure dyes were most pronounced, being more marked from the preparation with a low melting point and abnormal properties.

Following intraperitoneal injection all specimens caused death in a relatively short time. In each case 200 mg. dissolved in 3 cc. of warm oil were used. If death did not take place the same day the animal was fed on a mixture of the stained oil and dog biscuit. In all cases the abdomen when opened gave off a foul smell. The omentum and intestine both inside and out were loosely coated with the dye. A control experiment conducted with the plain oil proved harmless.

A number of rats after fasting for 4 days were fed specimens of the commercial dye dissolved in oil and mixed with dog biscuit powder. There was produced a general debility and appearance of malnutrition. Specimen 4 was unlike the other crude speci-

TABLE II.

Summary of Rat Experiments with Sudan III.

Experiment No.	Dye specimen.	Dose.	Administration of dose.	Effect.	Remarks.
		gm.			
14	3	0.2 in 3 cc. oil.	Intraperitoneal.	Died in convulsions.	
15	3	0.2 in 3 cc. oil.	Intraperitoneal and in food.	Paralysis of hind limbs.	Died after 1 wk.
16	2	0.2 in 3 cc. oil. 0.5.	Intraperitoneal. In food.	Very debilitated.	Died after 12 days; empty stomach; stained oil not all absorbed from the peritoneum.
17	1	0.2 in 3 cc. oil.	Intraperitoneal.	" "	Died in 2 wks.; lost quick movement; hind limbs very slow.
18	5 pure.	0.2 in 3 cc. oil	" "	Became bad tempered.	Continued to eat well; after 19 days gave Dye 4.
	4	*Ad libitum.*	In food.	Very debilitated.	Died 3 wks. later; loose hair; granulated ears; foul smelling, stained omentum and kidney fat.
19		Plain oil 3 cc.	Intraperitoneal.	No apparent effect.	After 1 wk. gave Dye 4.
	4	0.2 in 3 cc. oil.	"	Died in 3 days.	Stained oil mostly unabsorbed.
20	3	*Ad libitum.*	In food.	Debilitated.	Fasted 4 days, fed dye 3 days, then put on normal diet 1 mo., after which Dye 5 was given.

TABLE II—*Concluded.*

Experiment No.	Dye specimen.	Dose.	Administration of dose.	Effect.	Remarks.
		gm.			
20	5	0.2 in 3 cc. oil.	Intraperitoneal.	Died after 15 days.	
21	4	*Ad libitum.*	In food with oil for 1 wk.	Stunted growth, quite lively.	First fasted 4 days. During 8 wks. weight almost stationary, varying from 133 to 128 gm.
22	4	" "	" " "	" " "	Same as Experiment 21, varying from 168 to 153 gm. at time of death. Emaciated, very little body fat, no stained fat in the body.
23	3	" "	In food for 1 wk.	Debilitated, bad tempered till put back to normal diet.	Three rats grew normally up to nearly 300 gm. Killed after 8 wks. Omentum, neck, leg, and kidney fat heavily stained.

mens. The rats fed on it were very stunted in growth and did not show staining of the omentum and other fatty tissues of the body.

These experiments with the varied effects on the growth and health of the animal bring out the varied quality of the crude dye products, and confirm the results of the rabbit experiments which show the undoubted toxicity of the unpurified commercial preparations.

SUMMARY.

Sudan III dissolved in oil and administered intraperitoneally, subcutaneously, or by mouth, is absorbed, and may be traced in

the lymph, blood, and bile. It is transported to the fatty tissue of the body, being deposited particularly in the omentum. It is excreted in the feces, and is not found in the urine of normal animals.

Ordinary commercial preparations of Sudan III contain more or less impurity of a toxic nature. They may cause great debility, stunt the growth, produce a neuritic-like condition, and be fatal in large dose. The foreign substances may be excreted by the kidney, and appear in the urine as deep colored pigments.

The purification and properties of Sudan III are set forth, and their important bearing on problems associated with fat metabolism and vital staining discussed.

I am indebted to Professor Lafayette B. Mendel for assistance and suggestions in planning and carrying out the above experiments.

THE GLOBULINS OF THE JACK BEAN, CANAVALIA ENSIFORMIS.

PRELIMINARY PAPER.

BY JAMES B. SUMNER.

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PLATE 1.

(Received for publication, November 13, 1918.)

While working with the urease of the jack bean, the author has had occasion to study the nature of the proteins that are present. The proteins of the jack bean have been investigated by Jones and Johns,[1] who claim to have isolated an albumin and two globulins. The precipitate obtained by half saturation with ammonium sulfate, present in small amount and having the greater sulfur content, they called concanavalin. The precipitate by complete saturation with ammonium sulfate, present in large amount and containing less sulfur, was named canavalin. The only reason Jones and Johns had for considering the jack bean to contain two globulins was the different sulfur content of these two fractions. Jones and Johns dialyzed jack bean extracts and found that spheroids were formed.

The author has found not two but three globulins present in the jack bean. Two of these globulins crystallize readily upon dialysis, while the third appears to be incapable of crystallizing. These three globulins can readily be separated by virtue of their markedly different solubilities.

The greater part of the precipitate by dialysis consists of spheroids and represents the uncrystallizable globulin; this is readily soluble in 1 per cent sodium chloride solution. It also dissolves in distilled water when the acid combined with it has been neutralized. The author believes that the name canavalin, applied by

[1] Jones, D. B., and Johns, C. O., *J. Biol. Chem.*, 1916–17, xxviii, 67.

Jones and Johns to their precipitate by total saturation with ammonium sulfate, should be given to this protein.

The minute amount of material which crystallizes as needles is slowly soluble in 10 per cent salt solution and is not dissolved by alkali until an excess is present. The author has named this globulin concanavalin B.

The third globulin, crystallizing in bisphenoid form, and present in moderate amount, appears to be insoluble in any but concentrated salt solutions. The crystals when not recently formed probably have an outer layer of protean, and in this case will dissolve only in hot salt solution. If jack bean extracts are covered with toluene and simply allowed to stand exposed to the air for several weeks, this protein is precipitated as beautifully formed crystals having a diameter of about 0.1 mm. The author proposes to name this globulin concanavalin A.

When jack bean extracts are subjected to dialysis the bisphenoid crystals (concanavalin A) are precipitated first, canavalin separates next as spheroids, and the needles (concanavalin B) are formed last. A partial separation of the proteins can be made by filtering off the precipitates as they appear, but it is better to use the salt solutions named above. The author has never been able to accomplish any satisfactory separation by salting out with ammonium sulfate.

All three globulins give the usual protein color and precipitation tests when purified by several repetitions of dissolving and dialyzing; after such treatment the proteins give no Molisch test but may still contain phosphorus.

EXPERIMENTAL.

Extraction of Globulins.—The jack beans are ground as fine as possible in a coffee mill, and the meal is extracted over night with two and one-half times its weight of distilled water, toluene being mixed in as preservative. While salt solutions extract somewhat more of the proteins, the extra salt present considerably lengthens the time of the dialysis. The wet meal is wrapped in cheese-cloth and pressed in a tincture press. The milky juice is filtered by suction through a thick bed of filter paper pulp. A satisfactory filtrate can be obtained if the rim of the mat is firmly pressed down from time to time during the first part of the filtration.

Separation of Concanavalin A.—The filtrate is dialyzed against running distilled water, toluene being used as preservative in this as well as in all subsequent dialyses. The dialysis can be regarded as finished when a test portion of the material in the dialyser, on being filtered and added to distilled water containing a little dilute acetic acid, gives no haziness. The precipitate is filtered off by suction on an ordinary filter paper, weighed, and stirred to form a suspension with five times its wet weight of distilled water. Enough saturated sodium chloride solution is now added with stirring to give a salt concentration of about 10 per cent. The spheroid protein will dissolve at once, but the needles, the solubility of which depends upon size and age, may require as much as an hour for solution. Without waiting for this to take place, the material can be immediately started filtering by suction as the filtration will take a long time. The filter paper may clog up and have to be replaced by a new one.

The insoluble globulin remaining on the filter is washed with 10 per cent salt solution, transferred to a beaker, and dissolved with stirring in a very small amount of saturated sodium chloride solution at 40°C. Complete solution requires only a minute or so and can be observed by microscope. The denaturized protein present is removed by filtration through filter paper pulp contained in a small Buchner funnel. If the filtrate is diluted with distilled water and allowed to stand, or else is dialyzed, bisphenoid crystals will be formed.

Crystals can be obtained by dissolving concanavalin A in the least possible amount of dilute ammonium hydroxide and neutralizing cautiously with dilute acetic acid; large crystals are formed if the original jack bean juice is poured into distilled water acidified with acetic acid, and allowed to stand a day or so. Large and perfect crystals can be formed by allowing solutions of the protein containing considerable ammonium sulfate to evaporate slowly, but the best method of preparing well formed crystals is to allow the original bean juice to stand, covered with a thick layer of toluene, in an open cylinder for about 1 month. Presumably the carbon dioxide of the air is slowly absorbed by the protein solution, with the formation of the insoluble globulin salt. Fig. 1 shows crystals prepared in this manner. Dr. A. C. Gill has been kind enough to examine them, and has provisionally

declared them to be "bisphenoidal in shape, probably belonging to the rhombic system, optically biaxial, with a large optical angle, and negative in optical character."

Separation of Concanavalin B and Canavalin.—The filtrate from concanavalin A, containing the dissolved spheroids and needles, is dialyzed, and the precipitated proteins are filtered off by suction and suspended in a considerable amount of distilled water. Concentrated sodium chloride solution is slowly added with stirring until all the spheroid globulin has dissolved. The needles are separated by centrifuging, washed with a little 1 per cent salt solution, centrifuged again, dissolved in 10 per cent salt solution, filtered by suction, and again dialyzed. Under favorable conditions many of the needles formed by dialysis are large enough to be seen easily with the unaided eye, having a length of about 2 mm. When the needles are treated with a few drops of 0.1 N ammonium hydroxide, they split up transversely, and the microscope will reveal hexagonal cross-sections. Fig. 2 shows two of these cross-sections together with some of the partly broken needles.

With excess of ammonium hydroxide the needles dissolve completely and are reprecipitated on adding acetic acid. Mineral acids in small concentration dissolve the needles instantly, but in higher concentration cause coagulation. The needles stain well with methyl violet after the addition of a trace of ammonia.

The uncrystallizable canavalin dissolved in dilute salt solution is precipitated by dialysis, and can be purified further by redissolving in dilute saline, filtering through filter paper pulp, and dialyzing once more. Instead of dissolving the canavalin in salt solution, one can suspend it in distilled water and add dilute ammonium hydroxide drop by drop until the neutral point is reached. All the canavalin will dissolve before the reaction becomes alkaline. This behavior of canavalin shows it to be a globulin of the legume type.

CONCLUSION.

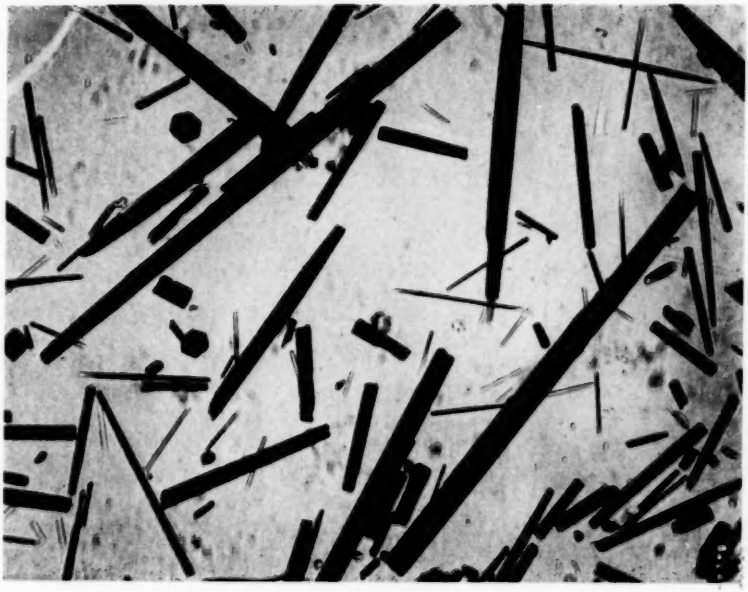
Two crystalline globulins and one non-crystalline globulin have been isolated from the jack bean. A method has been given for separating these globulins, and some of their properties have been described. More data will be published later.

The author wishes to express his thanks to Dr. B. F. Kingsbury for photographing the protein crystals, and to Dr. A. C. Gill for his kindness in pronouncing on the crystal form of concanavalin A.

EXPLANATION OF PLATE 1.

FIG. 1. Bisphenoids (concanavalin A). × 150. Stained with alkaline methyl violet.

FIG. 2. Needles (concanavalin B). × 135. Stained with alkaline methyl violet.



(Sumner: Globulins of jack bean.)

DO SEEDLINGS REDUCE NITRATES?

By J. DAVIDSON.

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(Received for publication, November 11, 1918.)

In the course of studies on the nitrogen economy in plant growth, particularly with reference to wheat, it was desired to obtain some quantitative measurements as to the extent to which wheat seedlings reduce nitrates to nitrites. It was assumed on the basis of the experiments of Laurent[1] with peas, corn, lupine, and rye, which were repeated by Schreiner and Sullivan on wheat,[2] that seedlings possess certain principles in the nature of reductases which reduce nitrates to nitrites.

Experiment I.

Wheat seeds were germinated on a large floating aluminum disk. The seedlings at the age of about 1 or 2 days were transferred to smaller paraffined wire netting disks to which corks were fastened to keep them afloat in small agateware pans, each containing 500 cc. of sodium nitrate solution of a concentration of 1,000 parts per million. There were eight pans, four with 50 and four with 100 seedlings. The determination of nitrites in the solutions was begun the next day after the experiment was started and was made colorimetrically approximately every 24 hours. The results are given in Table I. As seen from this table, the quantity of nitrites fluctuated from day to day without any regularity. There was no tendency for nitrite accumulation, but there was an intermittent rise and fall in the nitrite content.

[1] Laurent, M. E., Expériences sur la réduction des nitrates par les végétaux, *Ann. Inst. Pasteur*, 1890, iv, 722.

[2] Schreiner, O., and Sullivan, M. X., Studies in soil oxidation, *U. S. Dep't. Agric., Bureau of Soils, Bull. 73*, 1910.

All this could be explained by the complexity of the biological processes going on at the same time: simultaneous reduction and oxidation, reduction beyond the nitrite stage, bacterial activity, etc. It was noted that there was no correlation between the number of seedlings and the quantity of nitrite produced, but that there was distinct correlation between the nitrites and the extent to which the seed portion of the seedlings came in contact with the solution in the pans, due to the tilting of the disks in the solution. The more the disks were tilted and the more as a consequence the seed itself came in contact with the solution, the larger was the quantity of nitrites found in the respective solutions.

TABLE I.

Reduction of Nitrates by Seedlings Grown in Nitrate Solution on Floating Paraffined Wire Netting Disks.

Pan No.	No. of seedlings.	Nitrites in parts per million.									
		Jan. 30	31	Feb. 1	3	5	6	7	8	9	10
1	50	0.75	0.5	0.33	5.0	5.0	5.6	5.0	5.0	8.0	6.5
2	50	0.75	2.0	2.2	6.0	6.0	8.1	8.7	11.2	19.0	15.0
3	50	0.87	2.4	3.1	4.0	5.0	5.6	9.4	11.0	15.0	2.2
4	50	0.75	2.0	2.2	4.0	5.0	4.5	2.5	4.0	9.0	3.1
5	100	0.88	1.4	Trace.	3.5	6.0	5.0	2.2	2.5	4.0	2.4
6	100	0.88	0.8	"	2.0	4.0	2.8	5.0	4.5	12.5	13.1
7	100	0.66	1.4	"	10.0	9.4	9.2	16.2	16.0	16.0	2.7
8		1.0	4.0	2.7	7.0	10.6	11.2	4.5	6.0	17.5	6.0

This observation led the writer to reopen the question as to the seat of the reducing principle, which according to Laurent[1] was equally present in the seed and in the attached plantlet.

Experiment II.

Wheat seeds were germinated on a floating aluminum disk as before. 50 seedlings were placed in each of two 400 cc. beakers. 100 plantlets were severed from the mother seeds. 50 of the severed seeds were placed in each of two similar beakers, and 50 of the detached plantlets were likewise placed in each of two similar beakers. 200 cc. of sodium nitrate solution of a concentration of 1,000 parts per million and 5 cc. of toluene were added to

each beaker. Toluene was selected as an antiseptic because in another experiment with ungerminated seeds it completely prevented the formation of nitrites in a nitrate solution. The reduction of nitrates by ungerminated seeds is assumed to be due to bacterial activity.

The beakers were covered with watch-glasses and were allowed to stand at room temperature for 24 hours. The respective solutions were then tested qualitatively for nitrites. Appreciable quantities of nitrites were found in the beakers containing the whole seedlings and also in those containing the detached seed. No nitrites were found in the beakers containing the detached plantlets.

This led to the conclusion that the seat of the reducing principle, at least in wheat seedlings, is exclusively in the mother seed attached to the young seedling.

Experiment III.

This experiment was set up to obtain quantitative measurements. Particular precautions were taken in producing the necessary seedlings. The aluminum disk and the pans were immersed in a solution of bichloride of mercury (1:1,000) for about 2 minutes. The seeds were soaked in a solution of bichloride of mercury (1:1,000) for 20 minutes. The seed, pans, and disks were washed with sterile water. The pan was filled with sterile water, and a sterile pan was inverted over the pan containing the floating disk and the seed.

When the seedlings were ready for use they were gently washed by decantation with water saturated with toluene. The experiment was then carried out in the same way as in Experiment II, differing only to the extent that Erlenmeyer flasks instead of beakers were used as containers. The Erlenmeyer flasks were stoppered with cotton plugs and sterile rubber stoppers.

No nitrites were found in any of the solutions containing the detached plantlets, the detached seeds, or the whole seedlings. No nitrites were found in any of these solutions after standing a week. A determination of nitrates showed the amount of nitrate in the solution practically unchanged.

The only possible conclusion from this experiment was that wheat seedlings do not possess any principles reducing nitrates to

nitrites. The failure to produce nitrites could not be ascribed to the toluene, for when no extraordinary precautions were taken in obtaining the seedlings, the whole seedlings and the detached mother seeds did produce nitrites with toluene. It could not be ascribed further to the previous treatment of the seed with bichloride of mercury, since the seed did not fail to germinate and since this treatment did not prevent the production of nitrites under the experimental conditions of Laurent.

The nitrites produced in Experiment II were evidently due to bacterial action. It would seem as though the same might also be said of the experiments of Laurent and especially of the experiment of Schreiner and Sullivan since they worked with wheat. It is a fair assumption that their precautions were not sufficient to guard against bacterial contamination.

The question arises why toluene prevented the production of nitrites in a nitrate solution with ungerminated seed, and why it did not prevent the formation of nitrites in Experiment II. A possible explanation is that the reducing organism works its way into the interior of the storage products of the seed, and it is thus protected against the action of the antiseptic. Or it is possible that some of the organic products originally present in the reserve material or formed during the process of germination are exuded into the solution and protect the reducing organisms against the antiseptic action of the toluene.

Experiment IV.

The question further arose why the toluene prevented the formation of nitrites in the solution with the detached plantlets in Experiment II. Was it because they did not furnish any food materials for the reducing organisms or because they did not offer the hypothetical protection against the antiseptic mentioned above?

To answer this question Experiment IV was carried out. Growing wheat seedlings were placed in the nitrate solution in such a way as to prevent any contact of the seed attached to the seedling with the experimental nitrate solution.

Tumblers of about 250 cc. capacity were used. They were covered with paraffined paper in which small holes were made

with a pointed glass rod. The rootlets of the wheat seedlings, obtained from unsterilized seed and without any precautions, were introduced through holes in the paper covers into the tumblers filled with the sodium nitrate solution, so that the seed and plumule rested on the paraffined paper without coming in contact with the solution at all. No precautions of any kind were taken to prevent bacterial contamination during the entire period of the experiment, which lasted about a week.

Only slight traces of nitrites were developed during the entire week. These traces may be safely disregarded. The reason that suggests itself is that the reducing organisms could not develop in the solution owing to the fact that the roots did not furnish enough foodstuffs for their growth. This experiment showed more clearly than any of the others that growing wheat seedlings do not reduce nitrates to nitrites.

Experiment V.

The next natural step was to test the nitrate-reducing ability of some other seedlings. Seedlings of barley, corn, oats, rye, and rice were obtained in the manner described above. Some were obtained from seeds previously sterilized with bichloride of mercury, and some without previous sterilization. The experiment was carried out essentially as in Experiment III. There were four Erlenmeyer flasks for each cereal; two contained seedlings obtained from sterilized seeds and two seedlings from nonsterile seeds. In each case, to one of the two Erlenmeyer flasks toluene was added as an antiseptic, the other being allowed to stand without any antiseptic. The tests were continued for about 10 days.

The addition of toluene completely prevented the formation of nitrites in every case when the seedlings were obtained from sterilized seed, except in the case of rice where a maximum of 2 parts per million of nitrites was observed. The previous sterilization of the seed was probably less effective in the case of the rice, due to its tight seed coats. The addition of toluene to the seedlings obtained from seed not previously sterilized, did not prevent the formation of nitrites but inhibited it considerably. The nitrites formed ranged from 1.2 to 3.2 parts per million. The

highest amount was again formed by the rice seedlings. The previous sterilization of the seed without the addition of toluene prevented the formation of nitrites for 24 hours only in the corn, but it retarded the formation of nitrites in every case. The nitrites for the first 24 hours ran from 5 to 20 parts per million, while in the seedlings, which were obtained without previous sterilization of the seed, the nitrites ranged from 8 to 70 parts per million.

These data would suggest the general conclusion that growing seedlings do not, as a part of their metabolic processes, reduce the nitrates in the outside medium in which they are growing.

The writer is indebted to Dr. J. A. LeClerc for valuable suggestions.

GLOBULIN OF THE COCOANUT, COCOS NUCIFERA.

I. PREPARATION OF COCOANUT GLOBULIN. DISTRIBUTION OF THE BASIC NITROGEN IN COCOANUT GLOBULIN.

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The use of the cocoanut, *Cocos nucifera*, and cocoanut products has increased considerably in the United States in recent years. The fleshy endosperm is employed in a variety of ways by bakers and confectioners and also serves as a source of cocoanut oil. Before it was found necessary to divert tonnage for other uses, large quantities of copra were brought into this country for the preparation of cocoanut oil, and it is probable that its importation will be resumed as soon as the necessary tonnage is again available. Copra is prepared by removing the endosperm or cocoanut meats from the shells and then drying them in the sun or by means of artificial heat. It is then pressed to remove as much of the oil as possible. The residue or press cake, which contains about 20 per cent of protein (N × 6.25) is ground and used as a cattle food.

The principal protein of the cocoanut endosperm is a globulin which has been studied by Ritthausen (1), Kirkwood and Gies (2), Chittenden (3), and by Osborne and his coworkers (4). Osborne and Harris (5) studied the distribution of the nitrogen in cocoanut globulin by Hausmann's method and obtained the following results:

	per cent
Amide nitrogen	1.36
Humin "	0.14
Basic "	6.06
Non-basic nitrogen	10.92

So far as we are aware, no previous attempts have been made to ascertain the distribution of the amino-acids in cocoanut glob-

ulin. We have prepared the globulin and found the distribution of the basic amino-acids by Van Slyke's (6) method to be as follows:

Amino-Acid.	*per cent*
Cystine	1.44
Arginine	15.92
Histidine	2.42
Lysine	5.80
Tryptophane	Present.

The percentage of free amino nitrogen in the unhydrolyzed protein was also obtained using the method of Van Slyke and Birchard (7). This was found to be 3.68 per cent, which is in fair agreement with one-half of the lysine nitrogen (3.21 per cent), as found in the hydrolysate of the protein. The value for cystine is of course low since some of the cystine is decomposed during the hydrolysis of the protein with hydrochloric acid. These results indicate that cocoanut globulin contains all the basic amino-acids necessary for maintenance and growth. This assumption is borne out by the results of nutrition experiments in which cocoanut globulin was the only source of protein in the diet for a prolonged period during which normal growth was obtained. The nutrition experiments will be published later. A complete hydrolysis of cocoanut globulin is also in progress.

In order to obtain the large quantities of cocoanut globulin needed for hydrolysis and for nutrition experiments, it was necessary to devise a method that would not be too laborious or time-consuming. This method is described below.

EXPERIMENTAL.

Preparation of Cocoanut Press Cake.—The fresh cocoanuts were punctured and the milk was drained out. The shells were then removed and the endosperm was cut into slices. These were dried in a vegetable drying machine through which circulated a current of air heated to 50°C. The oil was then removed as thoroughly as possible by means of an oil press of the expeller type. The resulting press cake was ground to a coarse meal, which contained 21.5 per cent of protein (N × 6.25).

Preparation of Cocoanut Globulin.—About 2.5 kilos of the cocoanut meal were thoroughly mixed with 20 liters of a 10 per cent

sodium chloride solution and this mixture was allowed to stand for about a week at 1–3°C. in order to complete the extraction of the globulin. The mixture was then pressed in muslin sacks by means of a powerful press. The resulting turbid extract was again mixed with 2.5 kilos of meal and allowed to stand for a week at 1–3°C. The liquid was then pressed out as just described and filtered clear through paper pulp. The clear extract was dialyzed in parchment bags against running water for 7 to 10 days to remove chlorides. The globulin, which precipitated in a compact mass, was washed once by decantation with distilled water. It was then mixed with about 50 per cent alcohol and filtered off on a hardened filter, using suction. The globulin was then disintegrated and suspended in absolute alcohol. After standing for about 12 hours it was filtered off on a hardened filter and suspended in absolute ether. After standing in the ether over night it was filtered off as described above. The resulting globulin was placed in a vacuum desiccator over sulfuric acid to remove the ether. It was finally dried in a vacuum oven the temperature of which was gradually raised to 110°C. The protein was then ground to a fine powder in a mortar. The yield was 10 per cent.

Distribution of the Basic Nitrogen in Cocoanut Globulin by Van Slyke's Method.—Two 3 gm. samples of the protein were hydrolyzed for 28 hours by boiling each sample in 100 cc. of 20 per cent hydrochloric acid. Each sample was equivalent to 2.7957 gm. of moisture- and ash-free protein and contained 0.4860 gm. of nitrogen. The phosphotungstates of the bases were decomposed by the amyl alcohol-ether method (8). The results of the analyses are recorded in Tables I and II.

Determination of the Free Amino Nitrogen in Cocoanut Globulin.—About 2 gm. of freshly prepared protein were redissolved in 50 cc. of 5 per cent sodium chloride. The free amino nitrogen was determined in duplicate in the usual manner using the Van Slyke micro-apparatus. Caprylic alcohol was used to prevent foaming and a correction was made for the gas evolved by reagents used. The results are recorded in Table III.

TABLE I.

Analysis of Cocoanut Globulin (Van Slyke Method).

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0385	0.0391	7.92	8.05	7.99
Humin N adsorbed by lime	0.0063	0.0074	1.30	1.53	1.41
Humin N in amyl alcohol extract	0.0006	0.0004	0.12	0.09	0.11
Cystine N	0.0044	0.0049	0.91	1.01	0.96
Arginine N	0.1436	0.1432	29.55	29.46	29.50
Histidine N	0.0169	0.0189	3.48	3.89	3.68
Lysine N	0.0333	0.0291	6.84	5.99	6.41
Amino N of filtrate	0.2243	0.2174	46.15	44.73	45.44
Non-amino N of filtrate	0.0155	0.0292	3.19	6.01	4.60
Total N regained*	0.4834	0.4896	99.46	100.76	100.10

* Total nitrogen corrected for solubility of bases.

TABLE II.

Basic Amino-Acids in Cocoanut Globulin.

	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine	1.35	1.53	1.44
Arginine	15.94	15.90	15.92
Histidine	2.28	2.55	2.42
Lysine	6.19	5.41	5.80

TABLE III.

Free Amino Nitrogen of Cocoanut Globulin Compared with the Lysine Nitrogen.

Total N in 2 cc.	N gas from 2 cc.	Pressure.	Temperature.	Amino N in 2 cc.	Ratio of amino N to total N.	One-half lysine N by Van Slyke method.
mg.	*cc.*	*mm.*	°*C.*	*mg.*	*per cent*	*per cent*
12.01	0.76	759	24	0.424	3.53	3.21
12.01	0.82	757	23	0.458	3.83	

SUMMARY.

1. A detailed method for the preparation of cocoanut globulin is described.

2. The basic amino-acids of the cocoanut globulin were determined by the Van Slyke method of analysis.

3. The free amino nitrogen was determined and found to equal nearly one-half the lysine nitrogen as determined in the Van Slyke analysis.

BIBLIOGRAPHY.

1. Ritthausen, H., *Arch. ges. Physiol.*, 1880, xxi, 96.
2. Kirkwood, J. E., and Gies, W. J., *Bull. Torrey Bot. Club*, 1902, xxix, 321.
3. Chittenden, R. H., On digestive proteolysis, New Haven, 1895, 32.
4. Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, 1896, xviii, 609.
5. Osborne, T. B., and Harris, I. F., *J. Am. Chem. Soc.*, 1903, xxv, 323.
6. Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 15.
7. Van Slyke, D. D., and Birchard, F. J., *J. Biol. Chem.*, 1913–14, xvi, 539.
8. Van Slyke, *J. Biol. Chem.*, 1915, xxii, 281.

SUPPLEMENTARY RELATIONSHIPS BETWEEN THE PROTEINS OF CERTAIN SEEDS.

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It is well established that the proteins from different sources differ greatly in their values for the support of growth. Gelatin fails to maintain nitrogen equilibrium when it serves as the sole source of protein in the diet of an animal, and since this protein fails to give certain of the amino-acid color reactions which are recognized as tests for the presence of protein, the faulty character of gelatin from the dietary standpoint was correctly attributed to the lack of tryptophane, tyrosine, and cystine. The development of analytical methods for the approximate determination of several of the amino-acids which result from the hydrolysis of proteins led to the accumulation of evidence that there are great differences in the yields of the several cleavage products into which the food proteins are resolved in digestion, and accordingly the conclusion was accepted that there probably existed marked differences in the nutritive values of the proteins of our several natural foodstuffs. This was demonstrated to be true for a number of isolated proteins by Osborne and Mendel (1). They conducted experiments which showed that the isolated proteins from many different sources vary greatly in their nutritive value. Their diets were not so planned as to make the results a measure of the relative values of the proteins studied.

In each of the natural foodstuffs of both animal and vegetable origin, there always occurs a complex mixture of proteins which in most cases differ markedly from each other both in solubility and in the proportions of the different amino-acids which can

be obtained from them on hydrolysis. It seemed probable that such mixtures of proteins should supplement each other so as to make the differences in the values of the different kinds of food with respect to protein less marked than in the isolated proteins themselves, and experimental trial has supported this view. The protein mixture of any one of the seeds, tubers, and other vegetable foods, which have been studied by satisfactory methods, have been found complete biologically, in that they furnish when digested all the cleavage products which are required for the construction of new body tissues. The differences in the values of the different natural foods with respect to protein depend therefore on quantitative variations. We have studied the protein values of all the more important seeds (2), and the potato as a representative of the tubers (3), and can compare these with milk, which has proved to be one of the best sources of proteins of high quality (4). It is not possible by chemical methods to obtain data of sufficient accuracy for comparing the values of the proteins. This is true because there are several amino-acids which cannot be determined beyond simple qualitative detection.

The biological method for the analysis of a foodstuff which was described by McCollum and Davis in 1915 (5) made it possible to determine with considerable accuracy, not only the comparative values of the proteins in individual foodstuffs, but also the value of a food with respect to each of its dietary factors. The comparison of the proteins from different sources warrants the following conclusions.

1. The best proteins for the support of growth in the rat are those of milk and of eggs. Meats likewise apparently furnish proteins of relatively high value (6), but these have not been sufficiently investigated. Recent experiments by Drummond (7) indicate that the coagulable protein of fish muscle has a relatively high value for the support of growth.

2. The proteins of the cereal grains have approximately one-third to one-half the value of the proteins of milk for the support of growth or maintenance in the rat. Experiments with growing pigs indicate that as much as 63 per cent of the protein of milk can be retained for the construction of new body tissue whereas the proteins of the cereal grains can be retained only to the extent of 20 to 26 per cent (8).

3. The proteins of the pea (9) and the navy bean (10) have apparently about one-half the dietary value for the support of growth in the rat that has been observed for the proteins of the cereal grains. We cannot as yet state whether the exact value indicated for the proteins of the navy bean should be accepted as final. It is possible that a cause contributing to the animals' failure to develop was the extensive fermentation of hemicelluloses which are contained in the bean, and which beyond question caused injury to the alimentary tract through constant and extensive dilatation. This factor is not responsible for a false conclusion regarding the poor quality of the navy bean proteins, for we have fed beans at lower planes of intake and have in these experiments, in great measure, eliminated fermentation as a serious factor. These results confirm the view that the proteins are actually of low value, but possibly are slightly better than the growth curves indicate. The proteins of the soy bean are distinctly better than those of the navy bean or the pea, but soy bean proteins appear to be no better for the support of growth than are those of the cereal grains. The soy bean contains about three times as much protein as do the cereal grains and can be fed at high planes of intake without evidence of the presence of anything injurious, and therefore appears from the results of certain feeding experiments to be of excellent quality (11), but when fed in amounts which furnish such planes of protein intake as can be secured with cereal grains, and properly supplemented in all respects, the proteins of this bean appear to be of no greater biological value than are those of wheat, oats, or corn.

4. Certain proteins are deficient in one or more of the essential amino-acids and are, therefore, incapable, when fed as the sole source of protein, of inducing any growth whatever in a young animal. These proteins do, however, possess remarkable values as supplementary sources of amino-acids for certain vegetable proteins, and thereby greatly enhance their values for the support of growth. Thus McCollum and coworkers (12) found gelatin or zein to be of great value when fed with the proteins of the oat kernel. Neither are of appreciable value when fed as supplements to the proteins of the corn kernel. Gelatin is an excellent supplement to the proteins of the wheat kernel, but zein does not enhance the value of wheat proteins to an extent

which could be demonstrated by their experiments. Zein, however, supplements the proteins of the pea in a very satisfactory manner (9).

Osborne and his coworkers have described the results of the application of the Fischer ester method for the determination of the individual amino-acids to numerous proteins of both animal and vegetable origin (13). These values, while not in most instances actually quantitative in nature, show clearly that there are wide variations in the yields of the digestion products, the amino-acids in proteins from different sources. From such studies the deduction is warranted that when two or more natural foodstuffs, each of which contains proteins not possessing high values for the support of growth, are fed together, it is probable that they will in some degree supplement each other's deficiencies, because of one furnishing somewhat more of that amino-acid which in the other forms the limiting factor for the support of growth.

In former papers we have thrown some light on the supplementary relationship between the proteins of certain seeds (14) and between gelatin and the proteins of certain seeds (12). The present paper is descriptive of further experiments in the same direction. Chemical methods when applied to proteins have contributed greatly to the advancement of theory, but the data regarding the supplementary relationships among the proteins from different sources must be obtained by actual observations on growth or maintenance of animals. The biological method for comparing the relative values of the proteins from certain sources has yielded results very different from the results of chemical investigations of the same proteins. The proteins of the pea and navy bean appear from the quantitative chemical data available to be better constituted than are those of the important cereal grains, but feeding experiments on growing animals show the legume seeds to be inferior to these. In the present studies we have sought to find the relative values of mixtures of proteins derived from two seeds, one furnishing one-third and the other two-thirds of the total protein of the food mixture.

Our studies of the dietary properties of the more important seeds have shown that when each of these is supplemented with

purified food additions so as to make it satisfactory for the support of growth at the optimum rate, except with respect to the protein factor which is not supplemented, a content of protein equivalent to 9 per cent of the dry matter in the diet suffices to maintain growth at rates varying from less to more than half the rate at which the young rat is capable of growing. In such experiments as we are considering the seed is supplemented with respect to both certain salts and fat-soluble A, since it has been demonstrated that aside from the quality of the proteins as a limiting factor in the seeds these two are the only factors which require modification in order to complete the seed from the dietary standpoint.

In our studies relating to the special properties of our natural foodstuffs, we recognize the limitations of our experimental methods. When an animal in infancy is fed a diet unsatisfactory in any respect, it does the best it can under the circumstances. There is a variable factor in the vitality with which the individual is born, which in some measure will determine its ability to utilize food faulty in any respect. Only in this way can we account for the great variation in the ability of several individuals in the same experimental group to grow on the same diet. Since it is not possible to determine the extent to which any individual possesses natural vigor, an experiment should be conducted with several animals so that one or more will be of great vitality. The elimination of the unfit in the breeding stock is important for this reason.

When the faults of a diet are of a certain degree of magnitude, animals with a fair amount of vigor may grow at the normal rate and reach full adult size and yet not be in a state of optimum physiological well being. If continued on such diets, they may be tardy in maturing sexually, or may be capable of but a fraction of the fertility of the vigorous, well nourished individual. It has likewise become apparent that animals which have grown at a satisfactory rate, appear vigorous, and produce a few young at or near the usual age, may fail to maintain fertility to the age normal for the species. In all cases where the diet is below the optimum in character the signs of involution characteristic of old age appear earlier than in the well nourished. Since these several variations from the normal in the usual functions

of the adult animal serve as valuable criteria as to the state of nutrition and are more refined than the simple observation of the growth curve, we have extended our observations in recent years to include them.

Another test of great value in determining the adequacy of a diet is to observe the ability of the mother to nourish successfully a litter of young reduced to a standard number (15). The nutritive undertaking of the mother rat with about four young is sufficient to cause failure in weaning them successfully within 30 days if there is anything radically wrong with her diet. With minor degrees of deficiency from any cause the growth of the young will be retarded or suspended. In the present studies we have made observations on all these criteria, in order to estimate as accurately as possible the relative values of the proteins of the different rations in those cases where a comparison of the growth curves of the experimental animals would show no differentiation in the state of nutrition for animals of different groups.

The rates of growth secured on 9 per cent of protein from each of several seeds, properly supplemented with respect to inorganic salts and fat-soluble A, are shown in Chart 1. This chart shows that much better growth was secured with a ration similarly constituted but containing 8 per cent of protein from milk powder than with 9 per cent of protein from wheat, rye, maize, flaxseed, barley, oat, or kafir, and that in a mixture of rye and flaxseed, in which rye furnished protein equal to 6 per cent and flaxseed furnished half as much, protein was secured which has superior biological value to an equivalent amount of protein from any single variety of seed yet examined. Indeed it appears that rye and flaxseed proteins in this proportion are nearly if not quite equal in value for growth to the proteins of milk. This conclusion is tentative and must be confirmed by further reproduction records, as well as by observations on the rate at which animals fed comparable amounts of protein from the several sources here compared develop the characteristics of old age.

The value which such studies have both in human nutrition and animal production can be readily understood. We now fully understand the nature of the supplements which must be added to a mixture of seeds in order to render it complete from the dietary standpoint, and also that when such mixtures are supplemented

with respect to all other factors, the rate of growth will be determined solely by the extent to which the proteins of the food can be transformed into body proteins. It is therefore, of fundamental importance that we should know just what foodstuffs when combined give mixtures whose proteins make good each other's deficiencies, and to what extent, in order that we may make the best use of our foodstuffs. The character of the curves shown in the charts makes evident the extent to which economic loss may result from feeding wrong combinations of foodstuffs in animal production.

We have shown by numerous experiments, both with growing young and with adult animals, that a liberal supply of all dietary factors over maintenance requirements supports well being better than does a parsimonious supply (16). It is desirable to have definite information as to whether optimum nutrition can be secured equally as well with certain relatively low planes of protein intake, where the protein is of excellent quality, as with proteins of lower value fed in higher planes according to their relative worth for transformation into tissue proteins. To determine this we shall present data later on the comparative values of mixtures of proteins derived from multiple sources. It is not possible to answer the question as to how much protein we need for the most satisfactory promotion of human nutrition until we know the quantitative supplementary values of all the combinations of the important types of foodstuffs, and have determined by experiments upon animals the plane of protein intake of known relative value as compared with proteins from other sources, which most effectively defers the signs of old age.

Our object in feeding protein at the plane of intake of 9 per cent of the dry food mixture in these experiments should be explained. As previously stated, such cereal grains as maize, rye (9), and barley (9, 17) contain proteins of such values that when fed at 9 per cent of the food mixture, supplemented with respect to certain salts and fat-soluble A, young rats are able to grow at approximately half the normal rate. We prepared the present series of rations each containing 9 per cent of protein derived from two seeds, one furnishing two-thirds and the other one-third of the total protein, and supplemented them in the necessary ways. The results show whether the protein mixture

in any case has lower or higher values than maize, rye, or barley, and to what extent. This plan, as will be seen from a study of the growth curves in the charts, affords an excellent method for the quantitative comparison of a series of proteins with respect to their biological values. 2 per cent of butter fat we have found to be sufficient for the maintenance of good growth when all other dietary factors are of good quality.

In the series of experiments reported in this paper we have employed the following combinations of proteins:

Seed Furnishing 6 Per Cent of Protein.	*Seed Furnishing 3 Per Cent of Protein.*
Barley.	Flaxseed oil meal.
Wheat.	" "
Maize.	" "
Pea.	" "
Kafir.	" "
Soy bean.	" "
Rolled oats.	" "
Cottonseed flour.	" "
Millet seed.	" "
Rye.	" "
Soy bean.	Millet seed.
Wheat.	"
Rye.	"
Maize.	"
Rolled oats.	"
Pea.	"

The most interesting point brought out by the growth curves in these experiments is the failure of the proteins of two seeds to supplement each other to any greater degree than they do in most cases. According to the present universally accepted theory of protein metabolism, the explanation of this failure must be due to the relatively low yield of each of the seeds in a mixture in some one or more of the essential amino-acids. Until our experiments with the combinations of seeds are completed we cannot draw conclusions as to which amino-acids are the limiting factors in these experiments.

Chart 1.—These curves illustrate the rate of growth secured with young rats on diets containing 9 per cent of their dry substance as protein, derived in each case from a whole seed. The

dietary factors other than protein were so constituted as to be fairly satisfactory for the promotion of good nutrition. (The composition of these rations was seed = to 9 per cent protein; NaCl, 1.0; $CaCO_3$, 1.5; butter fat, 3.0; dextrin to 100). For comparison the growth curves of young rats on a diet containing 8 per cent of protein from milk and another its 9 per cent of protein from a mixture of rye and flaxseed are presented. Both the latter sources of protein are of distinctly better quality than are any of the protein mixtures derived from a single seed. It was determined in the case of each ration that the limiting factor was the protein supply, and that on the addition of purified protein and nothing else much better growth and nutrition could be secured.

With a ration containing 9 per cent of barley protein the growth curve was not so good as with a similar ration containing wheat protein. There were two females in the experimental group, and although both were kept until they were 8 months old, and showed distinct signs of age, no young were ever reared by them.

The growth curves for the animals fed 9 per cent rye proteins were somewhat better than were those for the barley group, but not quite so good as in the wheat group. Two females produced three litters of young (eighteen young). Two litters were allowed to die soon after birth. The other, consisting of eight young, fared somewhat better. At 10 days there were seven left (weight, 71 gm.). At 32 days but two were alive (weight, 55 gm.). Both of these survived to the 39th day. The mothers looked very old at the age of 8 months.

With the diet containing 9 per cent of maize proteins, one female was isolated in a pregnant condition, but no young were ever seen. She doubtless ate them as soon as they were born (2).

With wheat proteins at 9 per cent, only an occasional litter of one or two young has been secured (2). None of these was weaned.

The animals in these experimental groups only rarely showed soreness of the ears and tails. In nearly all cases they were nervous and irritable. Their coats were always poor, but there was little loss of hair, except in the case of the young which lived after the development of a coat of hair. All showed early appearances of old age.

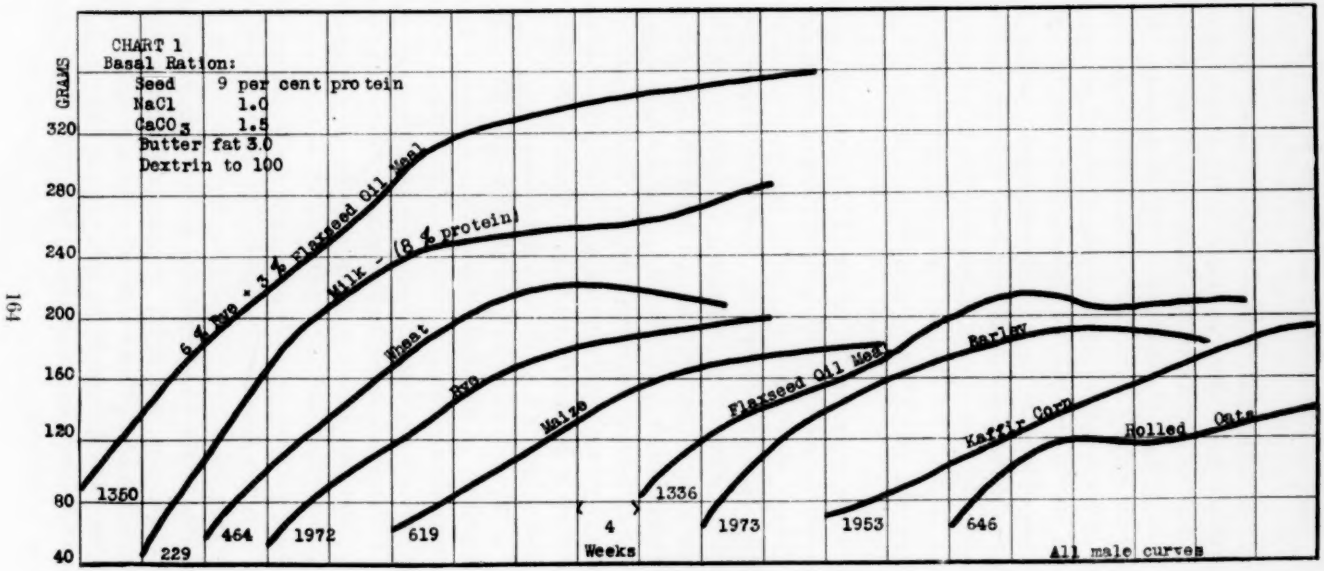
CHART 1
Basal Ration:
Seed 9 per cent protein
NaCl 1.0
CaCO3 1.5
Butter fat 3.0
Dextrin to 100
GRAMS
320
280
240
200
160
120
80
40
6 % Rye + 3 % Flaxseed Oil Meal
Milk - (3 % protein)
Wheat
Rye
Maize
Flaxseed Oil Meal
Barley
Kaffir Corn
Rolled Oats
1350
229
464
1972
619
4 Weeks
1336
1973
1953
646
All male curves

CHART 1.

In the experiments in which the curves for Lots 464, 619, and 646 were obtained, we employed complex salt mixtures instead of a simple mixture of sodium chloride and calcium carbonate, which we later found to be the only inorganic additions necessary for any of the seeds studied. These complex salt mixtures supplied, in the amounts in which they were employed, somewhat less calcium, sodium, and chlorine than we have later employed in supplementing these seeds. It is possible that with a more liberal supply of the three elements better nutrition might be secured with diets containing 9 per cent of wheat or maize proteins, but not sufficient to change in any marked degree the value of the comparisons made in this paper.

Each of these curves is representative of a group of four or five animals which were fed at the same time and in the same cage with the same ration. The curves show that a careful search for fortunate combinations of natural foodstuffs which will mutually enhance the values of each other's proteins, will yield results of importance both in human nutrition and animal production.

Chart 2.—Lots 1356 and 1350 illustrate the growth records of young rats fed a ration, the protein of which was derived from flaxseed oil meal (3 per cent) and rye (6 per cent). In the former case the mixture of seed products was supplemented with inorganic salts alone, and in the latter with both inorganic salts and butter fat to supply the fat-soluble A. The improvement resulting from the addition of butter fat is striking. The ration was, of course, of poor quality because of the character and amount of its proteins, and the improvement in well being which can result from supplementing a single dietary factor in a ration poor in some other respect emphasizes the principle which we have repeatedly enunciated; *viz.*, the importance of considering the relative values of the several factors which operate to make the diet what it is. In order to secure the best possible growth curves with any plane of protein intake which is just above the actual requirements of the animal, it is essential that the remaining factors in the diet be close to the optimum in quality. In many human diets derived from wholesome natural foods, two or three dietary factors are more or less faulty in character. Under these conditions there will be a lowering of the ability to

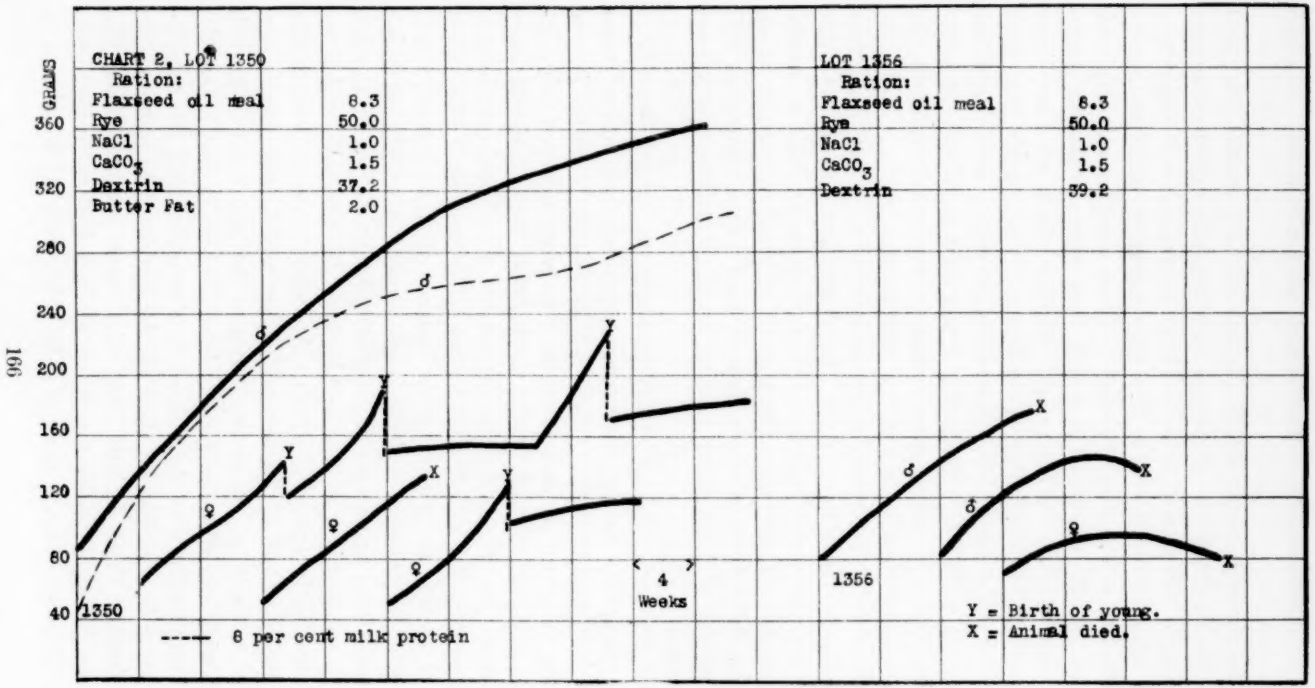
CHART 2, LOT 1350
Ration:
Flaxseed oil meal 8.3
Rye 50.0
NaCl 1.0
CaCO3 1.5
Dextrin 37.2
Butter Fat 2.0
LOT 1356
Ration:
Flaxseed oil meal 8.3
Rye 50.0
NaCl 1.0
CaCO3 1.5
Dextrin 39.2
GRAMS
360
320
280
240
200
160
120
80
40
1350
1356
4 Weeks
----- 8 per cent milk protein
Y = Birth of young.
X = Animal died.

CHART 2.

perform the normal functions of life, such as growth, reproduction, and the suckling of young as well as would be the case if all dietary factors were of better quality.

Both the growth and reproduction records of Lot 1350 were better than for any others in the series to which we have fed 9 per cent of protein derived from two seeds. No other group has been so successful in rearing the young.

Three females produced six litters (thirty-four young) of which ten young were successfully weaned. They appeared normal in vigor, but were small for their age. The mothers maintained their weight while nursing. One of the second generation died while giving birth to young. All were in good condition at end of the experiment.

Chart 3.—Lot 1339 derived 6 per cent protein from rolled oats and 3 per cent from flaxseed oil meal. Their growth was approximately that which might be expected had they received 9 per cent of protein derived entirely from one of the cereal grains, and corresponded to about half the rate at which the young rat is capable of growing.

The single litter of young born (seven young) were finally weaned but were undersized. At 56 days of age the seven together weighed but 313 gm. These young grew but little when confined to the diet of the mother. This further supports the view that there is a progressive loss of vitality under such a dietary régime as these animals were subjected to.

For comparison with the preceding lot, the curves of Lot 1336 are presented. These rats were fed 9 per cent of protein derived entirely from flaxseed oil meal. Two females produced each a litter of young. One mother destroyed her litter of six young soon after they were born. The other litter of four young weighed 107 gm. at the age of 34 days. They should have reached this weight when about 22 days old. The curves of two of this litter are shown in the chart. The young were not able to grow appreciably on this diet.

Lot 1344 received a diet containing 9 per cent of protein, 6 per cent of which was derived from millet seed and 3 per cent from flaxseed oil meal. Growth was very slow on this diet although it was fairly satisfactory with respect to all dietary factors other than protein. For comparison the curve of growth for a male

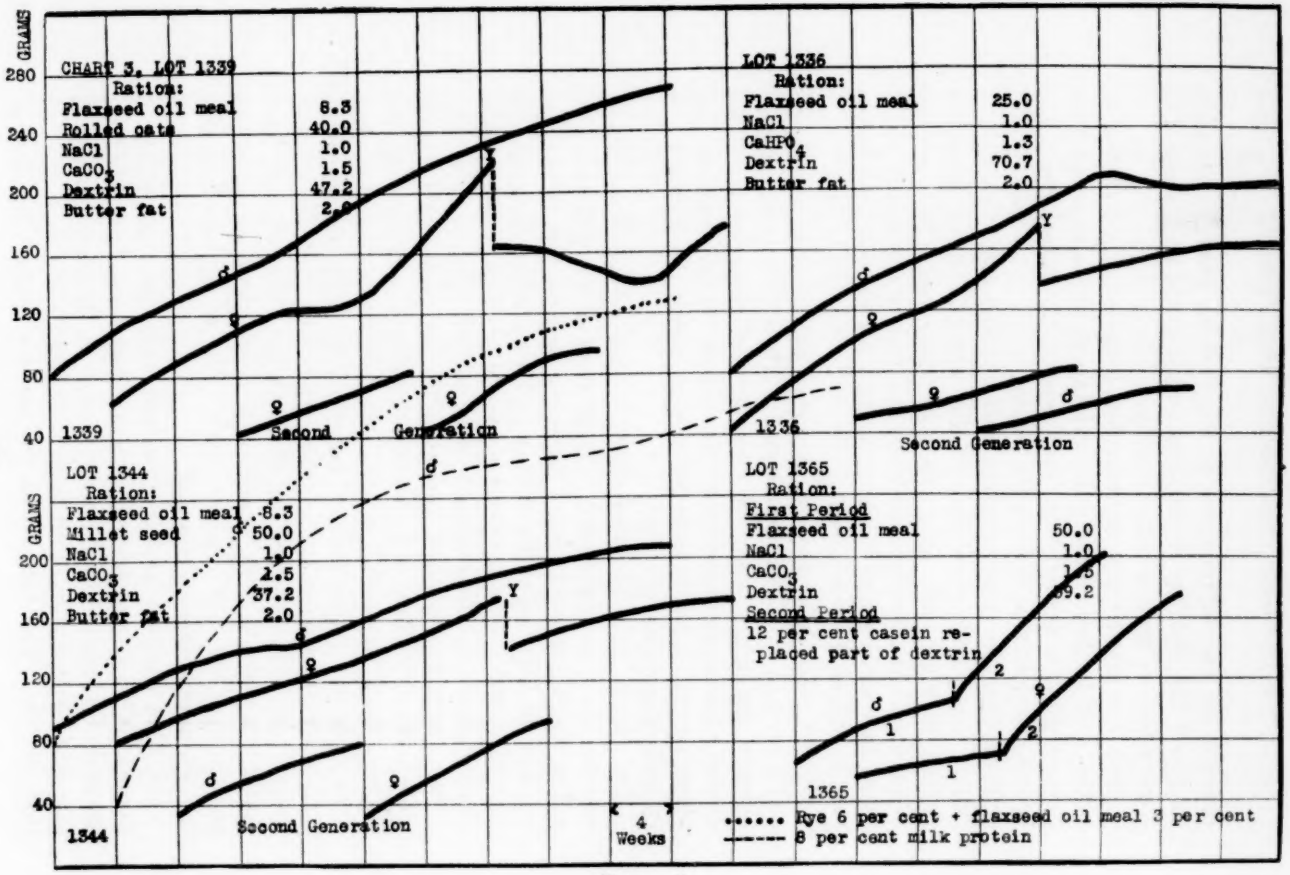
GRAMS
280
240
200
160
120
80
40
CHART 3. LOT 1339
Ration:
Flaxseed oil meal 8.3
Rolled oats 40.0
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 47.2
Butter fat 2.0
1339
Second Generation
LOT 1336
Ration:
Flaxseed oil meal 25.0
NaCl 1.0
$CaHPO_4$ 1.3
Dextrin 70.7
Butter fat 2.0
1336
Second Generation
LOT 1344
Ration:
Flaxseed oil meal 8.3
Millet seed 50.0
NaCl 1.0
$CaCO_3$ 2.5
Dextrin 37.2
Butter fat 2.0
1344
Second Generation
4 Weeks
LOT 1365
Ration:
First Period
Flaxseed oil meal 50.0
NaCl 1.0
$CaCO_3$ 1.5
Dextrin 39.2
Second Period
12 per cent casein replaced part of dextrin
1365
..... Rye 6 per cent + flaxseed oil meal 3 per cent
------ 8 per cent milk protein

CHART 3.

rat fed 8 per cent of protein derived from skim milk powder, and that secured with a diet containing 9 per cent of protein derived from rye and flaxseed oil meal, are placed in contrast with those of Lot 1344, and take the place of normal curves.

Lot 1365 was fed a diet containing 9 per cent protein and from the same source as in Lot 1344. In the case of Lot 1365 the grains were supplemented only with respect to inorganic salts, whereas in the diet of Lot 1344 both salts and butter fat (fat-soluble A) were added. The rate of growth was somewhat accelerated by the fat-soluble A. That the limiting factor in this diet was the character and amount of the protein is shown by the rapid growth in Period 2, when a part of the dextrin was substituted by 12 per cent of purified casein. Millet seed, cottonseed, or oat proteins are not supplemented by the proteins of the flaxseed to anything like the extent that rye proteins are.

Chart 4.—Lots 1343 and 1364 illustrate respectively the effects of feeding such a mixture of barley and flaxseed oil meal as would furnish a diet containing 9 per cent of protein, two-thirds of which came from barley and the one-third from flaxseed. In the one case it was supplemented with both inorganic salts and fat-soluble A (in butter fat), and in the other with inorganic salts only. The value of the protein mixture derived from barley and flaxseed in these proportions is not sufficiently high to enable the animals to grow on a diet containing 9 per cent of protein, at a rate faster than they could do on a similar diet in which the protein is derived solely from one of the cereal grains, as wheat or maize kernel. However, the reproduction records are in nearly all cases better on the mixed proteins than on a seed alone.

Two females in Lot 1343 gave birth to three litters of young (twenty-seven), and of these nine were weaned. No young were ever weaned on wheat or maize alone on diets of this character. Their growth was approximately half normal. Five young at 50 days of age weighed 260 gm. The mothers rapidly developed the characteristics of senility while nursing their litters.

In the second period of Lot 1364, there was a tardy response with renewed growth after the introduction of butter fat into the diet. Recovery is not always to be expected when the faults of a diet are corrected after suspension, because of lowered vitality in the animals.

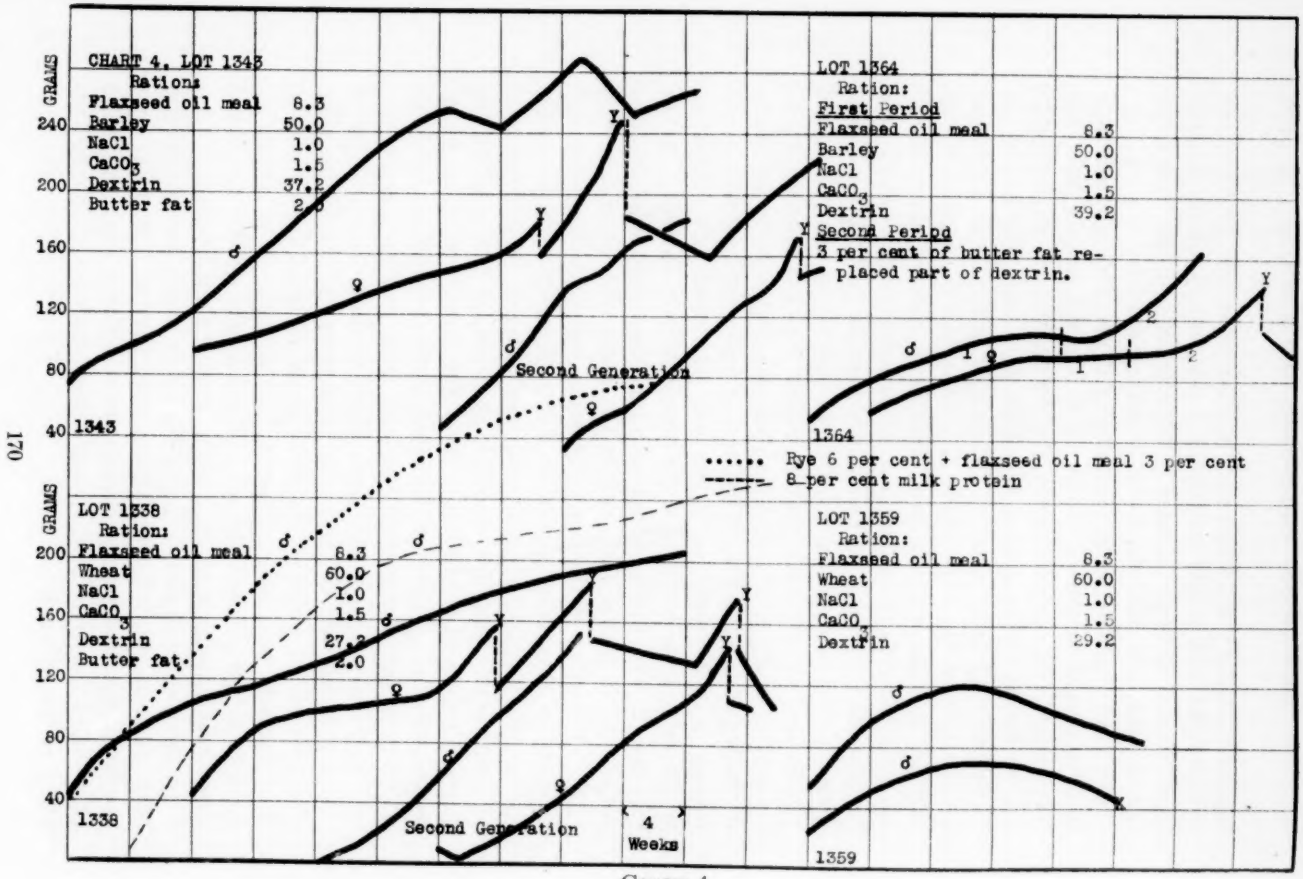
CHART 4. LOT 1343
Ration:
Flaxseed oil meal 8.3
Barley 50.0
NaCl 1.0
CaCO3 1.5
Dextrin 37.2
Butter fat 2.0
GRAMS
240
200
160
120
80
40
1343
Second Generation
LOT 1364
Ration:
First Period
Flaxseed oil meal 8.3
Barley 50.0
NaCl 1.0
CaCO3 1.5
Dextrin 39.2
Second Period
3 per cent of butter fat replaced part of dextrin.
1364
Rye 6 per cent + flaxseed oil meal 3 per cent
8 per cent milk protein
LOT 1338
Ration:
Flaxseed oil meal 8.3
Wheat 60.0
NaCl 1.0
CaCO3 1.5
Dextrin 27.2
Butter fat 2.0
1338
Second Generation
4 Weeks
LOT 1359
Ration:
Flaxseed oil meal 8.3
Wheat 60.0
NaCl 1.0
CaCO3 1.5
Dextrin 29.2
1359

CHART 4.

Lots 1338 and 1359 illustrate the great difference in the growth curves of two groups of rats which were fed diets containing but 9 per cent of protein, two-thirds of which came from wheat and the remainder from flaxseed oil meal, due to the addition of butter fat, as compared with what could be secured without it. With such rations it is possible to improve the nutrition of the animals either through the addition of protein or of fat-soluble A. The growth curves of young rats on 8 per cent of protein from milk and on 9 per cent protein from a mixture of rye and flaxseed (Lot 1350, Chart 2) are inserted as normal curves. Two females in Lot 1338 produced three litters (eighteen) young. Of these twelve were weaned. The nursing mothers lost weight during lactation. One dropped from 195 to 178 gm. in 44 days, during which time the young grew to a collective weight of 227 gm. The young were always small for their age and the mothers early showed signs of old age. That there is a fair supplementary value here is especially shown in the reproduction records. (Compare growth of second generation with Chart 3, Lot 1339 and Lot 1344.)

Chart 5.—Lot 1337 received a diet in which the content of 9 per cent of protein was derived from maize (6 per cent) and flaxseed oil meal (3 per cent). The diet was satisfactorily constituted with respect to all other dietary factors. Growth was slow, and the animals failed to reach the full adult size.

Two females were included in the experimental group. One of these never had any young, while the other had two small litters (six young), only two young of which were weaned, and these were small for their age. These animals were extremely timid and nervous.

Lot 1347, in Period 1, were fed a diet which was fairly satisfactory in all respects, except for the quality of its protein. The diet contained 9 per cent of protein, 6 per cent from peas (cooked and dried), and 3 per cent from flaxseed oil meal. In Period 2, 2 per cent of calcium phosphate was added to see whether the addition of more phosphorus would enhance the value of the diet. The results showed that this was not the limiting factor, but that the failure of the animals to grow at a better rate was the result of the insufficiency of the protein supply. The one litter of young produced were eaten by the mother. The de-

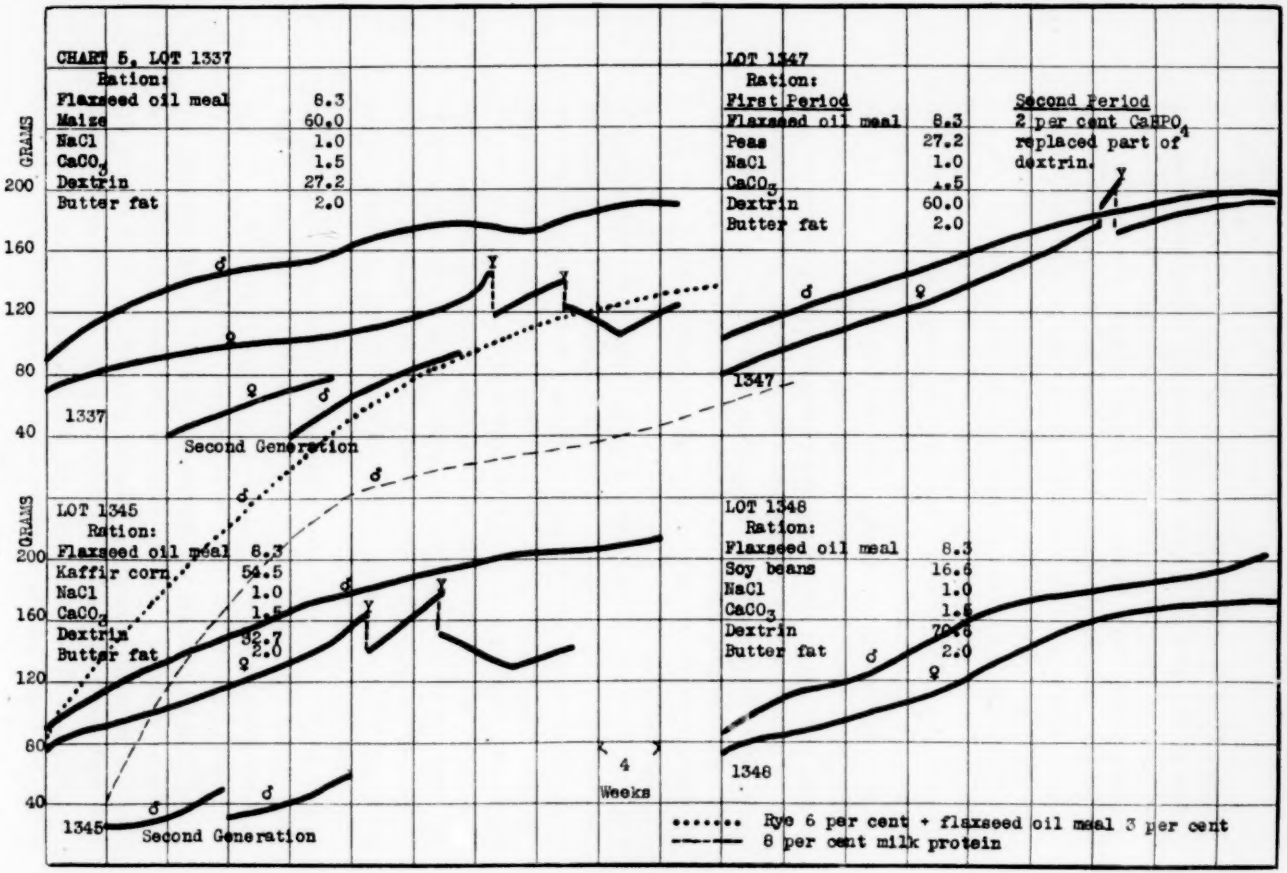
CHART 5, LOT 1337
Ration:
Flaxseed oil meal 8.3
Maize 60.0
NaCl 1.0
CaCO3 1.5
Dextrin 27.2
Butter fat 2.0
GRAMS
200
160
120
80
40
1337
Second Generation
LOT 1347
Ration:
First Period
Flaxseed oil meal 8.3
Peas 27.2
NaCl 1.0
CaCO3 1.5
Dextrin 60.0
Butter fat 2.0
Second Period
2 per cent CaHPO4 replaced part of dextrin.
1347
LOT 1345
Ration:
Flaxseed oil meal 8.3
Kaffir corn 54.5
NaCl 1.0
CaCO3 1.5
Dextrin 32.7
Butter fat 2.0
1345
Second Generation
LOT 1348
Ration:
Flaxseed oil meal 8.3
Soy beans 16.6
NaCl 1.0
CaCO3 1.5
Dextrin 70.6
Butter fat 2.0
1348
4 Weeks
Rye 6 per cent + flaxseed oil meal 3 per cent
8 per cent milk protein

CHART 5.

struction of the young by the mother rat is almost, without exception, the result of malnutrition.

Lot 1345 which was fed a diet containing 6 per cent of protein derived from kafir and 3 per cent from flaxseed oil meal failed to grow at any better rate than is possible on a similar diet in which the same plane of protein intake is furnished from one of the cereal grains. There is some evidence that the combination of the proteins from these two sources makes an improvement in their biological value. One female never produced any young, but the other had two litters (ten young). Five of these were successfully weaned, while the remaining litter was destroyed by the mother. All were very much stunted. At the age of 53 days they weighed collectively 127 gm. After weaning, the young never grew on the mother's diet.

Lot 1348 grew slowly on a diet in which the 9 per cent of protein was derived from soy bean (6 per cent) and flaxseed oil meal (3 per cent). No young were produced by this group. It is evident from the growth curves that this combination of proteins is of relatively low biological value for growth.

Chart 6.—Lot 1378 which was fed a diet containing 9 per cent of protein, two-thirds of which came from peas and one-third from millet seed, proved to be much better nourished than they would have been with the same amount of protein derived solely from one of the cereal grains. This is brought out especially by the ability of the second generation to grow as well as did the first, and to produce and rear young. Two females of the first generation, and a daughter of one of these produced six litters (twenty-five young) of which fifteen were reared. Although none of the females reached a non-pregnant weight greater than 175 gm., they were always in good condition. One female aged rapidly after the birth of her third litter of young. It is evident that we have in this combination of proteins from two seeds a supplementary relation of some importance.

Lot 1376, which derived its 9 per cent of protein from rolled oats (6 per cent) and millet seed (3 per cent), shows that again we have a supplementary relation between the proteins of one seed for those of the other, which distinctly enhances their value, but the improvement is less than in the case of a mixture of peas and millet seed (Lot 1378). Three females produced three litters

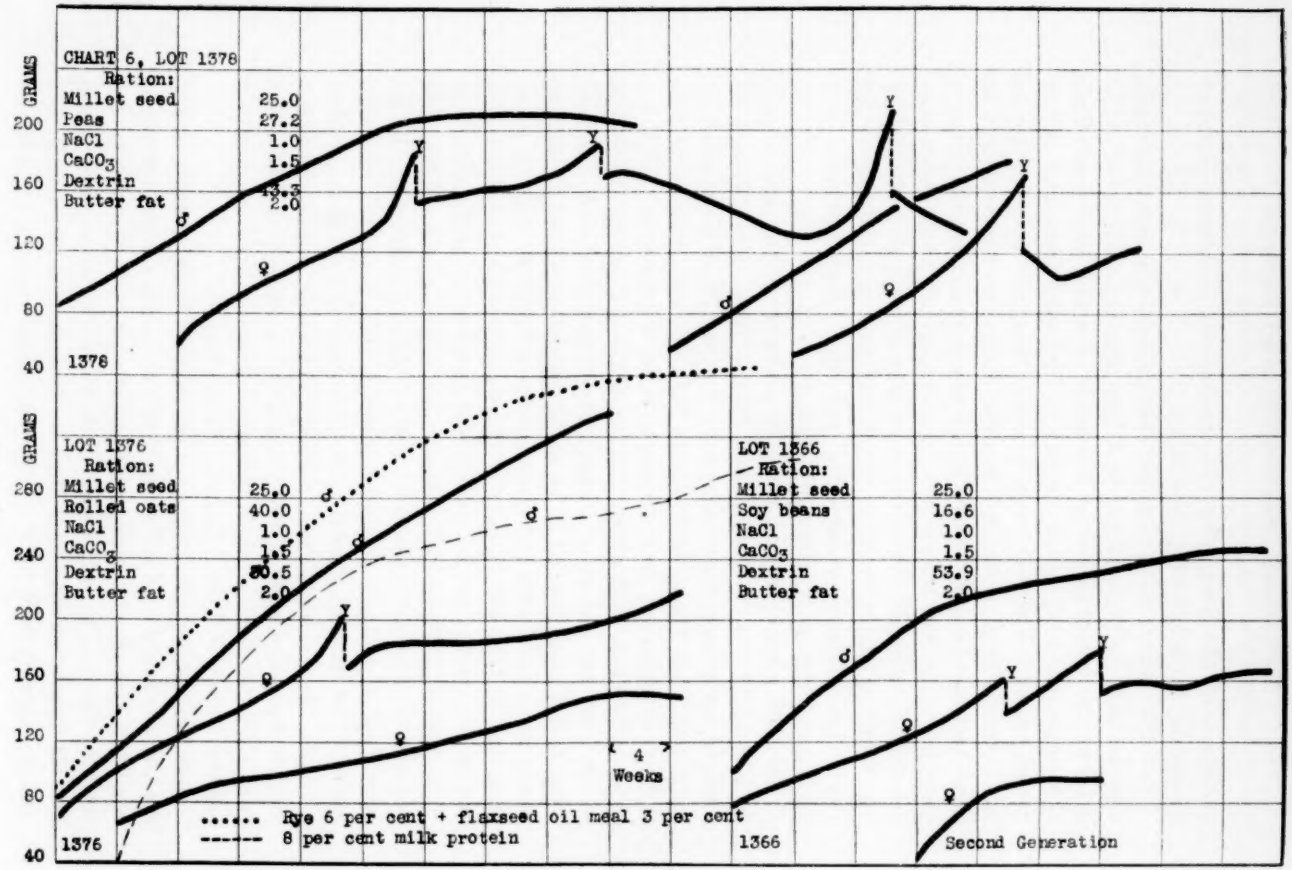
CHART 6, LOT 1378
Ration:
Millet seed 25.0
Peas 27.2
NaCl 1.0
CaCO3 1.5
Dextrin 43.3
Butter fat 2.0
GRAMS
200
160
120
80
40
1378
LOT 1376
Ration:
Millet seed 25.0
Rolled oats 40.0
NaCl 1.0
CaCO3 1.5
Dextrin 30.5
Butter fat 2.0
280
240
200
160
120
80
40
1376
LOT 1366
Ration:
Millet seed 25.0
Soy beans 16.6
NaCl 1.0
CaCO3 1.5
Dextrin 53.9
Butter fat 2.0
4 Weeks
..... Rye 6 per cent + flaxseed oil meal 3 per cent
------ 8 per cent milk protein
1366
Second Generation

CHART 6.

of young, all of which were destroyed by the mothers within the first 3 days after birth. The ability of different individuals to grow on this diet varied considerably, which is in harmony with our observations on faulty diets in general.

From the character of the curves of Lot 1366 it is evident that a mixture of the proteins of millet seed and soy beans when combined is little if any better than the proteins of wheat alone, or any of the other cereal grains alone. They are better, however, than we have been able to secure with 9 per cent of soy bean protein alone. Great variation in the capacity of different rats to grow on this diet was observed. There were three females in the group, and from these six litters were secured (thirty young). Ten of these were reared but were always undersized. The mothers all maintained their weights while nursing their young, which serves as evidence that this protein mixture was better than the proteins of one of the cereal grains taken as the sole source of protein.

Chart 7.—Lot 1374, which received 9 per cent of protein, two-thirds derived from wheat and one-third from millet seed, grew about as they would have on the proteins of one of the cereal grains alone at the same plane. The difference in the value of the proteins of this mixture as compared with those of the cereal grains alone is seen in the reproduction records. Whereas on wheat proteins at this plane of intake we have secured almost no young, an occasional litter of one to three being born, in the present case two female rats produced four litters (twenty-six young), and succeeded in rearing eighteen young. All remained undersized. We feel certain that such a reproduction record could not be secured with *this plane* of intake of proteins from any single cereal grain, satisfactorily supplemented in every respect except with regard to protein. The mothers in this group all maintained their body weights while nursing their young.

Lot 1375 derived its protein, which constituted 9 per cent of the diet, from rye (6 per cent) and millet seed (3 per cent), the mixture of seeds being properly supplemented with respect to other factors. The growth curves represent a little better than half the rate at which the young rat is capable of growing. Two females produced four litters of young (fourteen young) of which

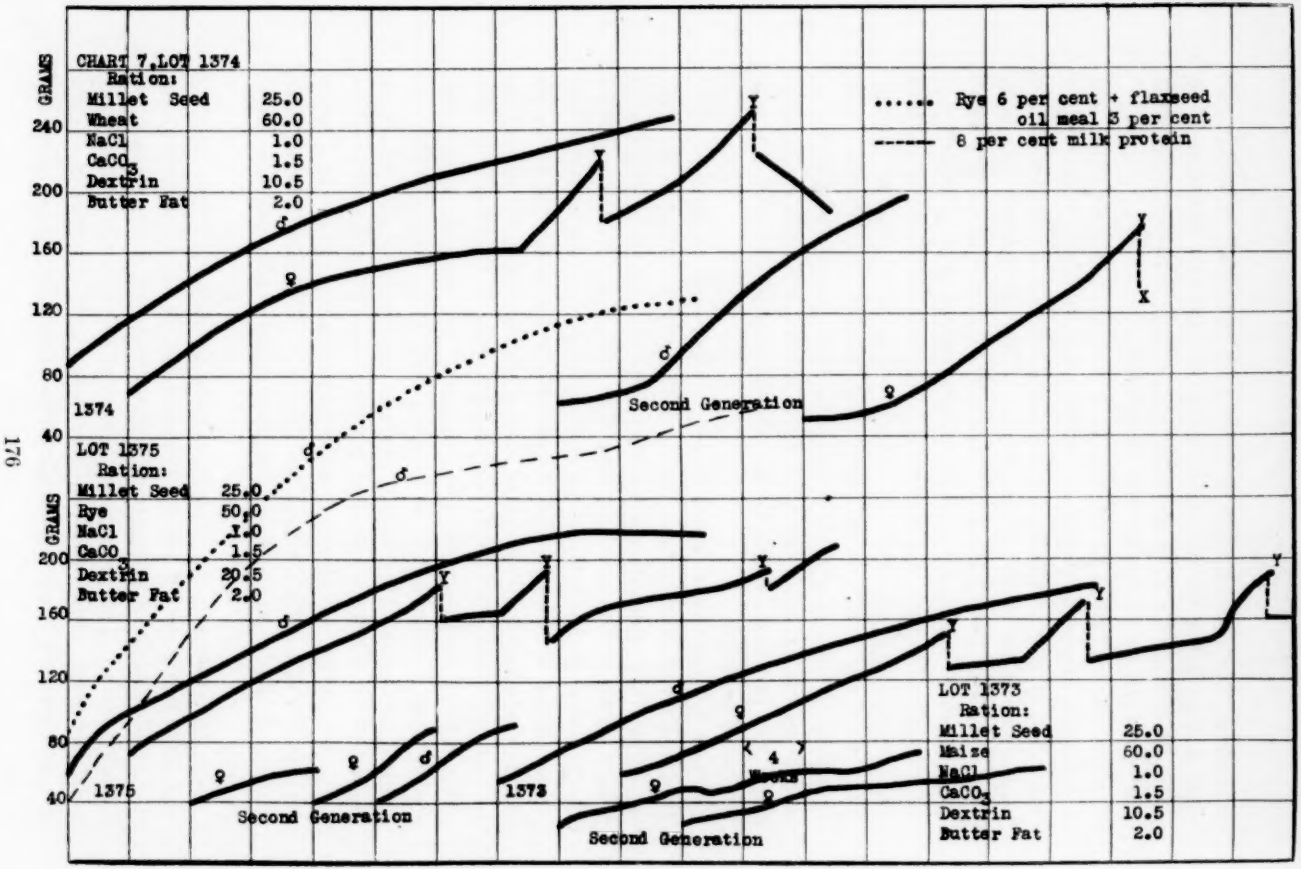
CHART 7.LOT 1374
Ration:
Millet Seed 25.0
Wheat 60.0
NaCl 1.0
CaCO3 1.5
Dextrin 10.5
Butter Fat 2.0
GRAMS
240
200
160
120
80
40
1374
Second Generation
Rye 6 per cent + flaxseed oil meal 3 per cent
8 per cent milk protein
LOT 1375
Ration:
Millet Seed 25.0
Rye 50.0
NaCl 1.0
CaCO3 1.5
Dextrin 10.5
Butter Fat 2.0
1375
Second Generation
1373
Second Generation
LOT 1373
Ration:
Millet Seed 25.0
Maize 60.0
NaCl 1.0
CaCO3 1.5
Dextrin 10.5
Butter Fat 2.0

CHART 7.

only three young were successfully reared, and these were undersized. The mothers lost weight while nursing their young and aged very rapidly. This evidence, together with the character of the growth curves, indicates that there is little enhancement of the proteins of one of these seeds by those of the other. The young, after weaning, were able to grow to some extent on the diet of the mother.

Lot 1373 was fed 9 per cent of protein from a mixture of maize (6 per cent) and millet seed (3 per cent). The growth curves indicate very little tendency, if any, of the proteins of one of these seeds to supplement those of the other. This is also supported by the reproduction records. Three females produced five litters (twenty-seven young) of which but three young were weaned and these were dwarfed by undernutrition. The mothers did not maintain their body weights while nursing their young. It appears, therefore, that there is little improvement in this mixture over the proteins of the individual seeds as the sole source of protein. All animals in this group aged early. Their young were almost hairless and very timid. None ever grew appreciably when confined to the mother's diet.

CHART 7.

BIBLIOGRAPHY.

1. Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication 156*, 1911, pt. ii; *J. Biol. Chem.*, 1912–13, xiii, 233; 1914, xvii, 325; 1914, xviii, 1, 95; 1916, xxv, 1; 1916, xxvi, 293; 1917, xxix, 69, 289.
2. Hart, E. B., McCollum, E. V., Steenbock, H., and Humphrey, G. C., *Wisconsin Exp. Sta., Research Bull. 17*, 1911. Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373. McCollum, E. V., Simmonds, N., and Pitz, W., *ibid.*, 1916–17, xxviii, 211. Hart and McCollum, *ibid.*, 1914, xix, 373. McCollum, Simmonds, and Pitz, *ibid.*, 1916–17, xxviii, 153. Hogan, A. G., *ibid.*, 1916, xxvii, 193. McCollum, E. V., and Davis, M., *ibid.*, 1915, xxiii, 181. McCollum, E. V., and Simmonds, N., *ibid.*, 1917, xxxii, 29.
3. McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1918, xxxvi, 197.
4. McCollum and Davis, *J. Biol. Chem.*, 1915, xx, 415.
5. McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 231.
6. Osborne and Mendel, *J. Biol. Chem.*, 1917, xxxii, 309.
7. Drummond, J. C., *J. Physiol.*, 1918, lii, 95.
8. McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 323.
9. McCollum, unpublished data.

10. McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxix, 521.
11. Daniels, A. L., and Nichols, N. B., *J. Biol. Chem.*, 1917, xxxii, 91. Osborne and Mendel, *ibid.*, 1917, xxxii, 369.
12. McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916–17, xxviii, 483.
13. Osborne, T. B., The vegetable proteins, Monographs on Biochemistry, New York and London, 1909.
14. McCollum and Simmonds, *J. Biol. Chem.*, 1917, xxxii, 29; 1918, xxxiii, 303.
15. McCollum and Simmonds, *Am. J. Physiol.*, 1918, xlvi, 275.
16. McCollum and Simmonds, *J. Biol. Chem.*, 1917, xxxii, 181, 347.
17. Steenbock, H., Kent, H. E., and Gross, E. G., *J. Biol. Chem.*, 1918, xxxv, 61.

STUDY OF THE METABOLISM IN MULTIPLE EXOSTOSES.

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That multiple exostoses are hereditary in character appears to be established by the observations of Jungmann (1), Gibney (2), Percy (3), and many others. It had not been clear, however, until recently that these tumors were congenital in character (Vaughan, 4), as they are not usually noticed until they attain an appreciable size. With the aid of the x-ray these growths have in many cases been shown to be present shortly after birth in children with exostotic parents. They are usually not noticed before the child is from 4 to 6 or more years of age, but they seldom, if ever, appear after the skeleton is fully developed. Males appear to be much more susceptible to the disorder than females (Fischer, 5). A female free of exostoses but belonging to an exostotic family may yield offspring which are affected. Ehrenfried (6) gives the ratio of males to females affected as 3:1.

There are a few cases on record where multiple exostoses were associated with rickets (Grünfeld, 7; Stolzenberg, 8), but this is not usually the case, and according to Vaughan there has been no demonstration of a direct connection between the two diseases. In fact it has been pointed out that the arrangement of the cartilage cells in growing exostoses is different from their arrangement in rickets. Close intermarriage has been observed by Meyer (9) in a case where no heredity of the disease could be proved, the father of the patient being the uncle of the mother. Ritter (10) noticed in one case that the thyroid gland was small and barely palpable. As this gland has an important influence on bone growth or at least on the metabolism of calcium, a closer observation of the condition of the thyroid in this disease may be

of interest. Lortat and Sebaréanu (11) and others have looked for a connection between tuberculosis and multiple exostoses. The tuberculosis theory, however, lacks any definite substantiation. As has been pointed out, tuberculosis is so common that there are sure to be some patients who have tuberculosis in addition to multiple exostoses. Recent authors consider the cause to be a disturbance in the bone-forming cartilage, though the real etiological factor is unknown. For discussions of the clinical aspects of the disease the papers of Vaughan and Ehrenfried may be consulted.

No attempts have been made so far as we are aware to determine by metabolic studies whether multiple exostoses are primarily a disorder of metabolism or whether the disorder shows from this standpoint any analogy to rickets. As a contribution to this phase of the subject, the present work was carried out on a patient in the Jefferson Hospital in the service of Dr. Hobart A. Hare, to whom we are indebted for the clinical history and for the opportunity of making this investigation.

Clinical History.—B. G. Female, age 15 years; native of Russia. Admitted to Jefferson Hospital, May 6, 1915, and discharged without change of condition June 26. Father, mother, eleven brothers and sisters all living and well. No history of cardiac, renal, pulmonary, chronic joint disease, or cancer in family. Patient never had any serious illness. Menses began at 14 years of age; about 2 months apart; no pain associated. Chief complaint related to the bony growths on the bones of the arms and enlargements of the finger joints. When the patient was 5 years old she began to notice an enlargement on the left radius which gradually increased. At 12 years of age there developed an enlargement on the middle finger of the right hand—middle phalanx. Some nodules appeared at about the same time on the ankles. At this time one of the nodules was removed from the radius.

The growth of these nodules was very slow and without pain. The only thing that the patient complained of was the deformity which went with the bony nodules. The patient was apparently suffering from a gastrointestinal disturbance also, as she passed 77 stools from May 15 to June 15, the number per day varying from one to eight. During the 7 days of the experimental period twenty-five stools were passed. This did not appear, however, to be a matter of discomfort or complaint.

Physical Examination.—Fairly well developed female. Eyes, ears, nose, mouth, and neck normal. Thorax fairly well developed. Abdomen normal, no tenderness and no masses felt. Extremities: There were a number of bony growths on the lower ends of the radius and ulna, with some thickening of bone generally. There was also a thickening of the digits with a tendency to dislocation due to atrophy of ligaments. There were some bony growths on the inner side of the ankles, similar to those on the radius and ulna. Weight: May 17, 84 pounds; May 24, 82 pounds; June 4, 81 pounds; June 11, 81 pounds; June 23, 81 pounds.

Method of Study.

The patient was kept in a ward of the hospital in charge of a special nurse and placed on a uniform diet which consisted of 500 cc. of milk, 90 gm. of soda crackers, 50 gm. of corn flakes, 100 gm. of meat, 30 gm. of potato chips, and 1,000 cc. of water per day. The milk was sampled every morning. The sample was poured into an evaporating dish, and evaporated at 100°C. Each succeeding day's sample was poured into the same dish, and at the end of the period the evaporation was continued to constant weight. The residue was ground up in a mortar and preserved in a glass stoppered bottle for analysis. Enough of the crackers, corn flakes, and potato chips were put aside for the whole period which necessitated but one sampling. The meat was also prepared for the whole period previous to the experiment, thoroughly mixed, boiled and again mixed, placed in glass jars, sterilized, and kept in a frozen condition until used. A carefully prepared sample was evaporated to constant weight and kept for analysis.

The urine was collected in exact 24 hour samples which were preserved by refrigeration and thymol. The feces were separated by the use of carmine. The stools as passed were collected in a friction top can which had previously been rinsed with a 10 per cent alcoholic solution of thymol and dried. The can containing the feces was kept in the refrigerator until the experimental period was over. As the sample was semiliquid in character because of water added in transferring, it was evaporated to dryness before analysis. The evaporation was carried out under

a pressure of 250 to 300 mm. and a temperature of 50–75°C. The gases coming off were first passed through standard acid and then through an alkaline lead solution to catch any hydrogen sulfide coming off. The total alkalinity of the gases calculated as ammonia nitrogen was 0.206 gm. or less than 0.4 per cent of the total nitrogen excreted during the experimental period. The lead sulfide formed was oxidized to lead sulfate and the sulfur estimated as barium sulfate. It amounted to 0.081 gm. of barium sulfate or 0.0113 gm. of sulfur, or less than 0.003 per cent of the total sulfur excreted. It is obvious, therefore, that the evaporation of feces did not incur the loss of an appreciable amount of these elements.

McCrudden's methods were followed for the determination of calcium and magnesium. Sulfur in the urine was determined by Benedict's method. Sulfur in the feces and the food was determined by a modification of the sodium peroxide fusion method which will be published later. Phosphorus was determined by Neumann's method, and nitrogen by the Kjeldahl method.

The experimental period lasted for 7 days, from May 29 to June 5.

DISCUSSION.

The most obvious abnormality brought out by the balance of the five elements (Table I) included in our study is the loss of 21.8 per cent of the nitrogen ingested. One might expect that this was entirely due to faulty digestion or absorption associated with the disturbed peristaltic action, but this cannot be the case as more than 87 per cent of the excretion of nitrogen was through the urine—a percentage not far from normal. It is obvious that the body is losing protein, which is borne out by the fact that the patient's body weight was slightly decreasing at this time, although the diet was higher in nitrogen than that of the free choice, preexperimental period. This loss of nitrogen and lack of appetite of the patient were no doubt associated with the gastrointestinal disturbance. It is somewhat surprising that the sulfur balance is positive, as sulfur excretion also commonly parallels protein metabolism. As the disease in question is characterized by an abnormal production of cartilage, which is known to be rich in sulfur, it is possible that the sulfur ordinarily set

free in the decomposition of body protein is retained for this purpose.

There is also a distinct retention of calcium, magnesium, and phosphorus. But the relative distribution in the excretion of these elements between the urine and feces is about normal. The calcium ratio for normal persons varies between 1 : 4.5 to 1 : 9, while we found 1 : 7.4. There is normally more magnesium found in the urine than in the feces, and we observed the same in this case; namely, 0.7988 gm. in the urine and 0.7552 gm. in the feces. From two-thirds to three-fourths of the phosphorus is found in the urine in normal persons, while we found 59.2 per cent in the urine, which is only slightly abnormal. It is interest-

TABLE I.

Metabolic Balance in Multiple Exostoses.

	CaO	MgO	P_2O_5	S	N
Total amount ingested, *gm.*	6.400	1.747	13.546	4.480	44.68
Excretion in urine, "	0.635	0.798	7.660	3.460	47.46
" " feces, "	4.695	0.755	5.286	0.661	6.99
Total excretion, "	5.330	1.554	12.946	4.121	54.45
Retention, *gm.*	1.070	0.193	0.600	0.4354	—
Loss, "	—	—	—	—	9.76
Retention, *per cent*	16.7	11.0	4.4	9.55	—
Loss, " "	—	—	—	—	21.8
Average daily gain, *gm.*	0.153	0.028	0.086	0.062	—
" " loss, "	—	—	—	—	1.39
Total excretion found in urine, *per cent*	11.9	51.4	59.2	83.9	87.2

ing to compare the per cent retention of these elements with those found by McCrudden and Fales (12) in a growing boy. We found that calcium, magnesium, and phosphorus were retained to the extent of 16.7, 11.0, and 4.4 per cent respectively, while McCrudden and Fales reported 17, 22.4, and 25 per cent respectively. It is not known at what stage in the development of the body the intake and output of these elements balance each other. It is not likely, however, that a balance would be reached at the age of 15, and as the ratio of the urine-feces excretion is approximately normal, it is probable that the metabolism of these elements is about normal. That absorption from the intestine was not quite so complete as usual was to be expected from the rapid-

ity with which the food residues passed through the bowel. Our metabolism study also supports the view that there is no connection between rickets and multiple exostoses as the urinary output of the ingested calcium has been found seldom to reach 5 per cent, more often being less than 1 per cent in rickets, while we find in multiple exostoses a normal excretion of 10 per cent.

Bergeim, Stewart, and Hawk (13) found pronounced daily variations in the urinary excretion of calcium and magnesium in their metabolism study in acromegaly and they suggested that this might be due to an accumulation in the intestine of residual material rich in salts which are soluble with difficulty. As in our study the intestines were well swept out, it is interesting to note that there was much more regular output of these elements. The

TABLE II.

Daily Excretion in Urine.

Day of experiment.	CaO	MgO	P_2O_5	S	N
	gm.	gm.	gm.	gm.	gm.
1	0.083	0.123	1.178	0.542	7.54
2	0.101	0.144	1.000	0.509	6.24
3	0.084	0.117	1.149	0.508	7.07
4	0.097	0.116	1.097	0.474	6.81
5	0.094	0.106	1.057	0.444	6.38
6	0.087	0.183	0.998	0.410	5.86
7	0.090	0.109	1.182	0.574	7.56

authors mentioned above (13) report an excretion of calcium oxide on 3 consecutive days of 0.190, 0.085, and 0.225 gm., and of magnesium of 0.250, 0.043, and 0.191, while our greatest variations for calcium for 3 consecutive days were 0.0828, 0.1012, and 0.0836 gm., and for magnesium 0.1229, 0.1441, and 0.1169 gm. (Table II).

A question naturally arises as to the existence of a relationship of the exostosis to the marked peristaltic activity of the intestine which might be expected to affect the general nutrition of the patient. This possibility may not be entirely ruled out by the failure of previous observers to note the presence of such disturbances in their cases, as, not being a matter of complaint in our patient, it might have been previously overlooked. It appears more likely, however, to be merely one of the numerous complications which have been found associated with multiple exostoses.

CONCLUSIONS.

A study was made of the metabolism in a case of multiple exostoses. A loss of nitrogen was noted and in part ascribed to the associated hypermotility of the intestine. A retention of sulfur was observed and attributed to storage in the growing cartilage. The retentions of phosphorus, calcium, and magnesium appeared to be within normal limits as did also the percentage excretions of these elements by way of the urine.

The findings do not support the view of a relationship of this disorder to rickets or to any primary disturbance of general metabolism.

The authors desire to thank Dr. P. B. Hawk for the opportunity of carrying out this work and for his suggestions.

BIBLIOGRAPHY.

1. Jungmann, *Berl. klin. Woch.*, 1902, xxxix, 890.
2. Gibney, V. P., *Am. J. Med. Sc.*, 1876, lxxii, 73.
3. Percy, *Surgery, Gynec. and Obst.*, 1915, xx, 619.
4. Vaughan, R. T., *Tr. Western. Surg. Assn.*, 1914, 115.
5. Fischer, B., *Ergebn. allg. Path. u. path. Anat.*, 1906, x, 697.
6. Ehrenfried, A., *J. Am. Med. Assn.*, 1917, lxviii, 502.
7. Grünfeld, R., *Münch. med. Woch.*, 1903, l, 277.
8. Stolzenberg, F., Inaugural dissertation, Greifswald, 1898.
9. Meyer, J. E., Inaugural dissertation, Würzburg, 1900.
10. Ritter, C., *Semaine méd.*, 1908, xxviii, 354.
11. Lortat-Jacob, L., and Sabaréanu, G., *Rev. méd.*, 1905, xxv, 119.
12. McCrudden, F. H., and Fales, H. L., *J. Exp. Med.*, 1912, xv, 450.
13. Bergeim, O., Stewart, F. T., and Hawk, P. B., *J. Exp. Med.*, 1914, xx, 218.

THE VITAMINES IN GREEN FOODS.*

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There is at present little available information which enables one to judge the relative amounts of vitamines furnished by the leaves of plants. Owing to the prevailing high cost of food products of this type the use of them may become greatly restricted, so that if they actually furnish an important part of the vitamine supply of our diet much harm may result from lack of them. Under present conditions, therefore, it is important to know the real nutritive value of the green foods in common use. If these serve no other purpose than to gratify the palate their use can be dispensed with without further concern, whereas if they furnish an essential addition to our dietary, care must be taken to supply a sufficient amount of them, and their cheapest sources should be ascertained.

In the case of the seeds of the more important cereals and of some of the legumes we already have information which affords an approximate estimate of the quantities needed to supply enough of the *water-soluble* vitamine for the normal growth of young albino rats. There are also similar data concerning numerous animal food products.[1] Some information respecting the

*The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[1] For references to the literature see Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 309; 1918, xxxiv, 17. Drummond, J. C., *J. Physiol.*, 1918, lii, 95.

distribution of the *fat-soluble* vitamine in food products of animal or vegetable origin has been obtained; but apart from indications of its relative abundance in the fats of milk, eggs, liver, and kidney, our present knowledge is still scanty.[1]

It has been stated that the green leaves of plants are especially rich in the fat-soluble vitamine[2] so essential for the growth of young animals, but the data on which this assertion is based are insufficient to justify quantitative conclusions of other than a very general character. For example, McCollum and his associates have shown that on a diet containing 10 per cent of immature alfalfa leaves as the source of all vitamines a young female rat grew slowly to 130 gm. during 5 months. Another young rat on a similar diet containing only 5 per cent of the alfalfa leaves lived for 2 months, but did not grow. When the quantity of alfalfa was increased to 30 per cent rapid growth and reproduction followed. Apart from statements by McCollum[2] that cabbage and clover furnish enough water-soluble vitamine for normal growth when the ration contains 15 to 20 per cent of the dried leaves, we have been unable to discover any further information relating to the content of this vitamine in green leaves, although numerous assertions have been made that they are especially rich in it.

In analyzing McCollum's data it is to be noted that while 20 per cent of dried alfalfa leaves permitted normal growth, 10 per cent led to slower growth and 5 per cent to none at all. We might, therefore, conclude that quantities between 10 and 20 per cent were sufficient to furnish enough of each of the vitamines needed; however, we cannot decide from these experiments whether growth was limited by a deficiency of the vitamine soluble in water or of that of the fat-soluble type. Consequently no opinion can be formed as to the richness of this plant in either one or the other of these essential food factors.

In view of the practical importance of definite knowledge of this subject and the paucity of available data we have under-

[2] McCollum, E. V., Simmonds, N., and Pitz, W., *Am. J. Physiol.*, 1916, xli, 333. McCollum, E. V., *Harvey Lectures*, 1916–17, xii, 151; *J. Am. Med. Assn.*, 1917, lxviii, 1379. McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxx, 13.

taken to study this question. Owing to the urgent need for such information we have been led to publish this preliminary account of the results thus far obtained. More detailed reports including reference to numerous other vegetable products will follow.

When any natural food contains more than one type of essential accessory it is necessary to test for each one separately if the relative content is to be ascertained. Thus in order to learn the minimum amount required to furnish enough of the fat-soluble vitamine for growth, the product to be tested must be fed in combination with a diet rich in water-soluble vitamine; and *vice versa* the minimum quantity required to furnish an adequate supply of the water-soluble vitamine for growth can only be determined when the basal diet contains sufficient of the fat-soluble type.

As a preliminary to such an investigation it is important to know whether the methods employed in preparing the various substances to be tested have any deleterious effects upon their vitamines. The initial content of vitamines in fresh green leaves cannot be established owing to their high water content, which makes it practically impossible to feed definite quantities during the long periods involved in properly conducted experiments. Therefore it is difficult to obtain direct evidence of changes which may be caused by drying. Such evidence as we have, as well as that reported by others, indicates that the water-soluble vitamine is stable at temperatures below 100°, and perhaps even at considerably higher temperatures. Thus it has been shown[3] that wheat embryo, milk whey, and crude lactose from which the vitamine has not been fully removed, can be heated for 1 hour at 15 pounds pressure without apparent deterioration of the water-soluble vitamine, and we can confirm the statement that protein-free milk can be boiled for several hours without destroying its activity as a source of this essential food accessory. McCollum, Simmonds, and Pitz[4] have also found that navy beans still retain much, if not all, of their value as a source of water-soluble vitamine after being heated in a moist condition at 15 pounds pressure for $1\frac{1}{4}$ hours. In addition we have shown that butter fat through which live steam was passed

[3] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 247.

[4] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxix, 521.

for 2½ hours was still efficient as a source of the fat-soluble vitamine,[5] but that the butter "oil" lost its potency if it was allowed to stand at room temperature for several months.[6] Recently Steenbock and his coworkers[7] have maintained that very prolonged heating of butter fat tends to diminish and eventually to destroy its value as a source of fat-soluble vitamine. In their behavior towards heat, the water- and fat-soluble vitamines must not at present be confused with the so called antiscorbutic factor in foods, which seems to be quite easily destroyed by even moderate heating.

Preparation of Plant Products.

The freshly gathered green leaves were carefully freed from adhering impurities, cut into small pieces, and dried at about 50–60° in the air, and then finely ground. The preparation of spinach included both the leaves and stems; that of cabbage only the sound inner leaves, which were separated from the stalk; those of the young alfalfa, clover, and timothy grass included the entire plant as obtained by mowing. These latter were cut in May when the alfalfa was about 20 inches high, the clover about 10 inches, and the timothy about 24 inches. All these plants were green and growing vigorously when cut.

Spinach Leaves as a Source of Water-Soluble Vitamine.

Chart I shows the results of feeding an otherwise adequate diet in which both the water-soluble and the fat-soluble vitamines were supplied solely by 10, 5, or 1 per cent of dried spinach leaves.

Those animals which received 10 per cent at first grew at a normal rate, but after 2 or 3 weeks grew more slowly. After being fed on this diet for 70 to 90 days the four animals responded promptly with a more rapid rate of growth when 200 mg. of yeast per day were furnished instead of spinach as a source of the water-soluble vitamine. From these results we conclude that 10 per cent of spinach supplies somewhat less than

[5] Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 381.

[6] Osborne and Mendel, *J. Biol. Chem.*, 1916, xxiv, 37.

[7] Steenbock, H., Boutwell, P. W., and Kent, H. E., *J. Biol. Chem.*, 1918, xxxv, 517.

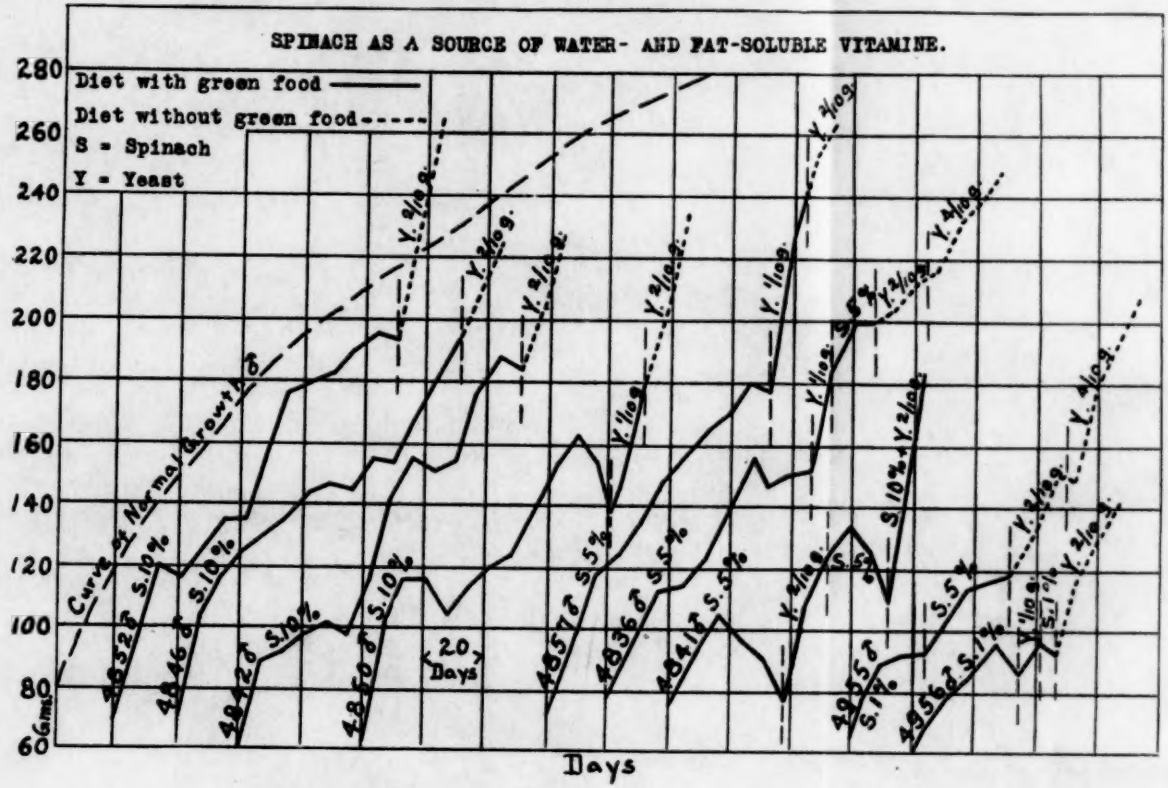
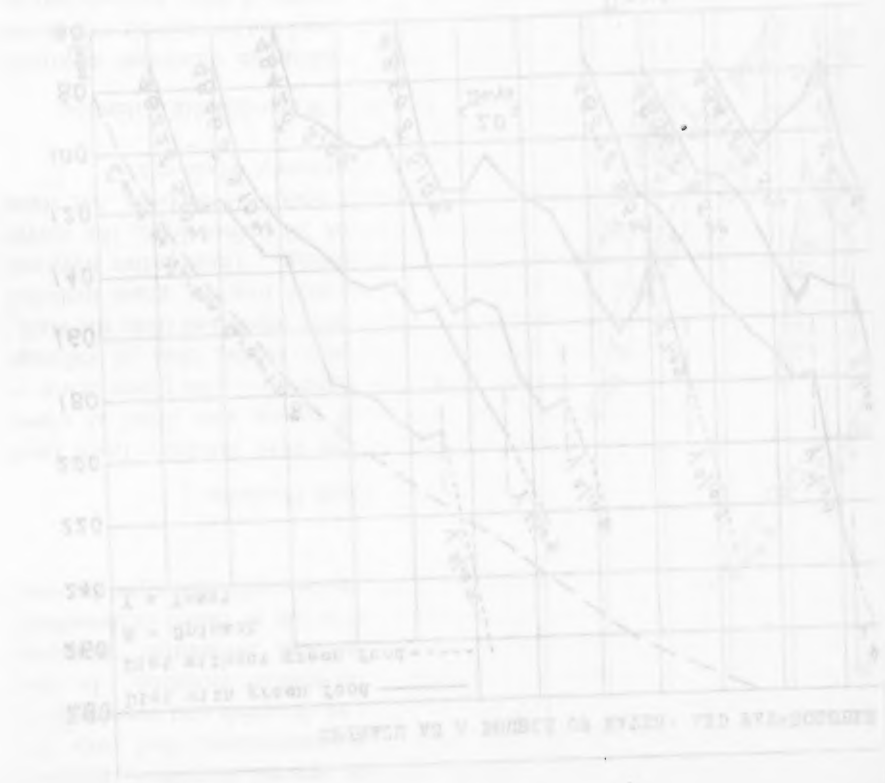


CHART I.

	per cent	per cent	per cent	per cent
Spinach	10.0	5.0	1.0	0.0
Meat residue	18.3	19.0	19.0	19.6
Salt mixture*	2.0	2.0	2.0	4.0
Starch	44.7	49.0	53.0	52.4
Lard	25.0	25.0	25.0	24.0

* The preparation of this mixture is described in *J. Biol. Chem.*, 1913, xv, 317.



enough of the water-soluble vitamine needed for normal growth. Unless the food intake is known, experiments of this kind afford no information respecting the absolute amount of the vitamine-containing constituent of the food needed by the normally developing animal. The quantity actually consumed by the rats in these experiments can be estimated from Table I. From these figures it appears that from 4 to 7 gm. of dry spinach were eaten per week, or a total of 66, 71, 72, and 63 gm. respectively during the 13 weeks in which the rats received spinach. According to our experience about 1.4 gm. of dried brewers' yeast per week are required to promote normal growth, or 18 gm. during 13 weeks. Dried yeast is therefore somewhat more than four times as efficient as dried spinach leaves when used to supply water-soluble vitamine. We have already found that when used as a source of water-soluble vitamine the quantity of whole wheat, soy beans, dried eggs,[8] or milk solids[9] required to promote normal growth is approximately twice as large as is the quantity needed of the dried spinach.

When only 5 per cent of dried spinach furnished all the vitamine, practically normal growth was made during the first 2 weeks. Growth then became slower and ceased much sooner than when 10 per cent was fed. That the rats receiving 5 per cent of spinach were suffering from a deficiency of the water-soluble vitamine is shown by their prompt recovery when yeast was added to their ration. The failure of Rat 4841 to continue growth as long as Rats 4836 and 4857, on the same diet, is due to the fact that after the 1st week it ate very much less food than the other two and consequently received proportionately less of the spinach (see Table I). Its subsequent rapid recovery when supplied with more vitamine shows that on a suitable diet it was able to gain weight with great rapidity. When the food contained only 1 per cent of dried spinach growth soon ceased (Rats 4955 and 4956). Rat 4955 resumed growth for a short time when the quantity was increased to 5 per cent, and both these rats grew steadily after yeast was substituted for the spinach.

The marked difference in the curve of body weight for Rat 4841 compared with curves of the others on the 5 per cent spin-

[8] Osborne and Mendel, unpublished data.

[9] Osborne and Mendel, *J. Biol. Chem.*, 1918, xxxiv, 537.

TABLE I.

Weekly Food Intake, in Gm., of Rats on Diets Containing Spinach.

Rats....4852	4846	4842	4850	4857	4836	4841	4955	4956
Period 1 Spinach 10% 60 74 46 46 46 48 55 63 48 43 44 46 40	Period 1 Spinach 10% 69 68? 48 49 48 53 47 50 56 44 57 59 64	Period 1 Spinach 10% 56 57? 69? 70 35 51 70 62 42 39 69 62 44	Period 1 Spinach 10% 66 71 54 28 42 44 41 46 55 52 35? 34	Period 1 Spinach 5% 52 60 49 48 56 53 52 49 46 44	Period 1 Spinach 5% 45 59 51? 50 52 57 46 49 36	Period 1 Spinach 5% 46 43 35 32 27	Period 1 Spinach 1% 49 38 27	Period 1 Spinach 1% 42 38 34 28
Period 2 Yeast 0.2 daily	Period 2 Yeast 0.2 daily	Period 2 Yeast 0.2 daily	Period 2 Yeas 0.1 daily 72	Period 2 Yeast 0.1 daily Spinach 5% 86	Period 2 Yeast 0.1 daily Spinach 5% 94?	Period 2 Yeast 0.2 daily Spinach 5% 64 52	Period 2 Yeast 0.0 daily Spinach 5% 33 36 35 32	Period 2 Yeast 0.2 daily* 30
			Period 2 Yeas 0.1 daily 72			Period 3 Yeast 0.0 daily	Period 3 Yeast 0.2 daily	Period 3 Yeast 0.0 daily† 24

Spinach 0% 107 108	Spinach 0% 103 73	Spinach 0% 84 88	Period 3 Yeast 0.2 daily Spinach 0% 92 95	Period 3 Yeast 0.2 daily Spinach 0% 94 82	Period 3 Yeast 0.0 daily Spinach 5% 66 65 Period 4 Yeast 0.2 daily Spinach 0% 72 84 Period 5 Yeast 0.4 daily Spinach 0% 84 96 98 95	41 52 Period 4 Yeast 0.2 daily Spinach 10% 91	Spinach 0% 47 60 Period 4 Yeast 0.4 daily 72 82 84 92	Period 4 Yeast 0.2 daily Spinach 0% Lost.

*During last 4 days.
† " " 2 "

ach diet, illustrates occasional differences encountered in feeding individual animals on identical diets containing a fixed proportion of the vitamine-bearing ingredient. If for any reason the food intake of an animal falls below the normal, the amount of vitamine consumed likewise falls, with the effect of impairing the condition of the animal and further reducing its food intake. Thus it goes from bad to worse, for the influence of the vitamine is continuously diminishing and consequently also the food intake. This is by no means an isolated example, for our records contain numerous cases of this character. Table I, which gives the amount of food eaten each week during the various periods of these experiments, shows how great an increase in food intake results when the supply of vitamine is increased, the absolute amount eaten usually being doubled or trebled. The importance of an adequate supply of vitamine is especially exhibited by the four rats on the 10 per cent spinach diet. During Period 1 these rats were already receiving enough vitamine to promote growth at a nearly normal rate. The increased vitamine supply furnished in Period 2 very greatly increased the quantity of food eaten (see Table I).

Whether the water-soluble vitamine simply increases appetite and hence promotes growth, or whether it supplies some essential chemical factor which renders growth possible cannot be determined by any data heretofore recorded. The fact remains, however, that the addition of vitamine to a ration containing too little of this factor is followed by a very marked increase in food intake with accompanying rapid gain in weight. On the other hand, loss of weight on vitamine-poor diets almost invariably precedes a lessened intake of food, from which it seems more probable that the impaired health of the animal is due to insufficiency of an essential food factor rather than to an insufficient quantity of food. Conversely, when the water-soluble vitamine is increased in quantity after a decline on a vitamine-poor diet the increased food intake which follows is most likely due to an improved physical condition caused by the vitamine, for the resulting rapid recovery in body weight must necessarily be accompanied by a correspondingly greater food intake.

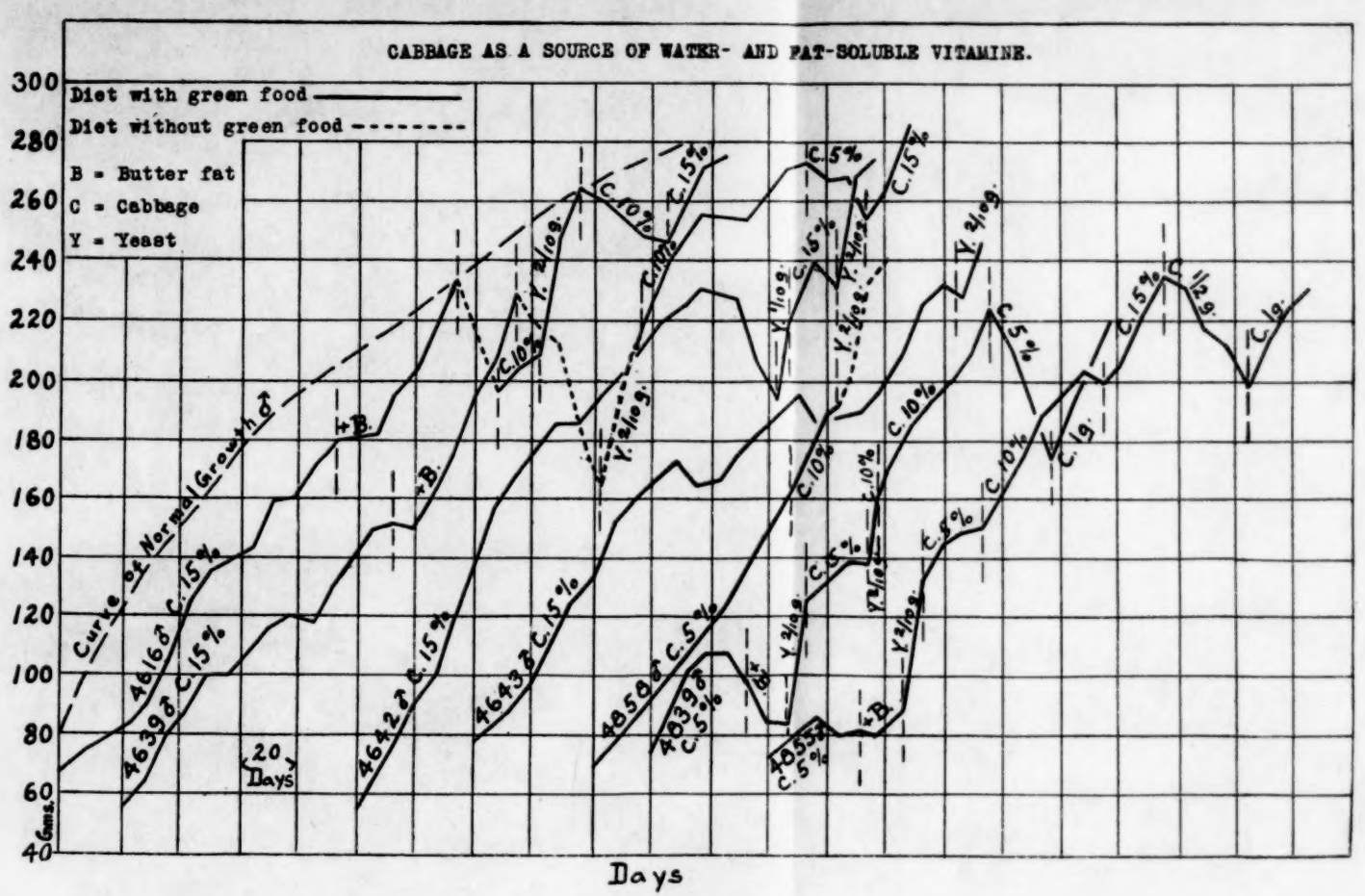


CHART II.

	Rats 4616, 4639	Rats 4642, 4643	Rats 4616, 4639	Rats 4838, 4839, 4855		Rats 4616, 4639	Rats 4858, 4839, 4855
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Cabbage.....	15.0	15.0	10.0	10.0	5.0	0.0	0.0
Meat residue.....		18.3		21.0	20.5		19.6
Casein.....	16.0		17.5			18.0	
Salt mixture*	2.0	2.0	2.0	2.0	2.0	5.0	4.0
Starch.....	42.0	39.7	45.5	42.0	47.5	50.0	52.4
Lard.....	25.0 or 16.0	25.0	16.0	25.0 or 16.0	25.0 or 16.0	18.0	24.0
Butter fat....	0.0 or 9.0		9.0	0.0 or 9.0	0.0 or 9.0	9.0	

* The preparation of this mixture is described in *J. Biol. Chem.*, 1913, xv, 317.

Fat-Soluble Vitamine in Spinach Leaves.

It is too early to make final statements as to the relative amount of the fat-soluble vitamine in spinach leaves, but the experiments here described indicate that these are rich in this food factor, so important for the normal growth of young rats. Even after 190 days of experimental feeding the four rats supplied with the 10 per cent spinach have shown no lack of the fat-soluble factor in their diet, as evidenced by a decline of weight or affected eyes, although after the first 80 to 90 days of feeding the spinach was removed from their food and nothing else supplied an appreciable amount of this type of vitamine. Rat 4852 at the present time has attained a weight nearly 50 per cent above normal while the other three are of practically normal weight. Rats 4836 and 4857, similarly receiving only 5 per cent of spinach, or 34 and 26 gm. during the first 87 and 83 days respectively of the 180 days of experimental feeding, are still normal in weight, although they received none of the fat-soluble vitamine during the last 93 and 97 days respectively.

These facts indicate that spinach leaves are in reality richer in this food factor than most of the products used in our ordinary rations. The final outcome of these experiments, together with others now in progress, must show how small a quantity will actually supply all that the young rat needs for normal growth.

Water-Soluble Vitamine in Cabbage Leaves.

Chart II shows that when 15 per cent of dried cabbage supplied both the water-soluble and the fat-soluble vitamines growth ensued at slightly less than the normal rate. It is possible that the failure of Rats 4616 and 4639 to make better growth was due to a deficiency in the fat-soluble factor, for when 9 per cent of butter fat replaced a corresponding quantity of lard an appreciable change in the rate of growth occurred. However, this failure was more likely due to a deficiency of water-soluble vitamine, because Rats 4642 and 4643, also receiving 15 per cent of cabbage, continued to gain in weight for a much longer time and recovered, after they had ultimately failed, when a little yeast was added to their diet. This recovery occurred in spite of the fact that no butter fat was fed during the 180 days of the experi-

TABLE II.

Weekly Food Intake, in Gm., of Rats on Diets Containing Cabbage.

Rats....4616	4639	4642	4643	4858	4839	4855
Period 1	Period 1	Period 1	Period 1	Period 1	Period 1	Period 1
Cabbage	Cabbage	Cabbage	Cabbage	Cabbage	Cabbage	Cabbage
15%	15%	15%	15%	5%	5%	5%
41	38	51	49	43	61	37
48	52	54	48	44?	52	32
40	52	51	52	44	42	26
44?	54	58	57?	45	29	30
47	42	62	57	44		
61	42	69	64	47	Period 2	Period 2
53	42	67	60	55	Cabbage	Cabbage
51	37	62	60	47	5%	5%
51	36	64	52	47	29	35
65	37	51	42		27	31
57	45	62	50	Period 2		
69?	45	72	58	Cabbage	Period 3	Period 3
73?	54	75	68	10%	Yeast	Yeast
		73	67	67?	0.2 daily	0.2 daily
Period 2	Period 2	68	69?	64	Cabbage	Cabbage
Cabbage	Cabbage	76	52	70	5%	5%
15%	15%	70	66		61	69
61	41	57	63	Period 3		
60	57	44	60	Yeast	Period 4	Period 4
82?	61.		73	0.2 daily	Cabbage	Cabbage
77	79	Period 2	83			
87	86		72			
			58			

95	95	Yeast 0.1 daily Cabbage 15% 74	Period 2 Yeast 0.2 daily Cabbage 15% 96	Cabbage 0% 85 89	5% 44 37 38	5% 50 53 38
Period 3 Cabbage 0% 74 49 Period 4 Cabbage 10% 58 52	Period 3 Cabbage 0% 68 55 28 29?	Period 3 Cabbage 15% 79 51			Period 5 Yeast 0.2 daily Cabbage 10% 62	Period 5 Cabbage 10% 66 53 60 61 63 62
Period 5 Yeast 0.2 daily Cabbage 10% 94 96	Period 4 Yeast 0.2 daily Cabbage 0% 73 84	Period 4 Yeast 0.2 daily Cabbage 15% 93 86			Period 6 Cabbage 10% 67 64 69 69 92	Period 6 Cabbage 15% 70 87 93
Period 6 Cabbage 10%	Period 5 Cabbage 10% 93 78 80 80				Period 7 Cabbage 5% 65	Period 7 Cabbage 0.5 daily

TABLE II—*Concluded.*

Rats... 4616	4639	4642	4643	4858	4839	4855
84	97				41	54
101?	92				35	46
79?	96					47
102?					Period 8	32
	Period 6				Cabbage	
Period 7	Cabbage				1 daily	Period 8
Cabbage	5%				59	Cabbage
15%	82				68	1 daily
96	68				61	56
103	69					65
91						
	Period 7					
	Cabbage					
	15%					
	92					
	91					

mental feeding. With yeast alone, even when much larger quantities were administered we have never succeeded in restoring rats which were declining on diets low in the fat-soluble vitamine.

When the ration contained only 5 per cent of dried cabbage Rats 4839 and 4855 soon declined in weight and failed to recover when provided with an abundant supply of butter fat. Since both these rats recovered very rapidly when a little yeast was added to the diet the failure to grow was obviously due to an insufficient supply of the water-soluble vitamine. Chart II shows plainly that 15 per cent of cabbage affords somewhat less than the minimum of water-soluble vitamine needed for normal growth. Expressed in terms of absolute intake the rats which grew on 15 per cent of cabbage ate 85, 86, 119, 107 gm. respectively of dried cabbage in 13 weeks; an amount not quite sufficient to supply enough water-soluble vitamine to promote growth at the normal rate. It will be noted that quantities of dried spinach somewhat smaller than those of cabbage sufficed to secure normal growth. However, one must be careful not to draw final conclusions from too few experiments, for during the first 9 weeks of experimental feeding Rat 4858 on the 5 per cent cabbage diet made as large a gain in weight as the average gain made by the rats on the 15 per cent diet although the absolute quantity of cabbage eaten by this rat was only about one-third of the average amount eaten by the rats on rations containing the larger proportion of cabbage. This is an example of the capacity of an animal to grow well, although receiving only a relatively small amount of vitamine. We have already called attention to this subject in connection with the effect of yeast as a vitamine.[10]

Water-Soluble Vitamine in Alfalfa, Clover, and Timothy Plants.

All these preliminary experiments with alfalfa, clover, and timothy plants in their rapidly growing state were made with too small quantities to enable us to make statements of definite value in regard to their content in water-soluble vitamine or to compare them with spinach or cabbage leaves (see Chart III). The results which were obtained with clover and timothy are similar

[10] Osborne and Mendel, *J. Biol. Chem.*, 1917, xxxi, 157.

to those recorded for comparable proportions of cabbage leaves. The growth made on diets containing only 5 per cent of alfalfa indicate that this plant is somewhat richer in water-soluble vitamine than either clover or timothy. New tests, now in progress, must show the effect of feeding larger quantities of these food products than were used in these experiments.

Fat-Soluble Vitamine in Alfalfa, Clover, and Timothy Plants.

The striking feature of these experiments is the small amount of these plants needed to supply sufficient *fat-soluble* vitamine for long continued and vigorous growth. Thus in spite of the deficiency of *water-soluble* vitamine supplied in the first period all these rats at the time of writing, after 170 days of experimental feeding, are still at a weight practically normal for their age although fat-soluble vitamine was furnished to none of them, except Rat 4908, from any other source than the green plants. During the entire course of the experiment they have eaten only 26 and 32 gm. of alfalfa, 23, 24, and 23 gm. of clover, and 25, 21, and 20 gm. of timothy. More experiments now in progress are needed to show how much less than this small quantity will furnish enough fat-soluble vitamine to suffice for normal growth. We have not given the food intake of these rats fed with alfalfa, clover, or timothy, because such data have no special importance in the present discussion.

If one may draw conclusions from the limited data now available, it seems that the green vegetables supply an important addition to the diet of man because the staples such as cereals, meats, potatoes, fats, and sugar probably furnish too small an amount of either of these vitamines to meet fully the requirements of an adequate dietary. Therefore care should be taken not to reduce greatly the quantity of green vegetables customarily eaten until more is learned about the actual requirements for these food factors and their relative abundance in the commonly used vegetables and green foods. Only then will it be safe to apply the results obtained in the laboratory to attempts to effect economies in the use of these relatively expensive food products.

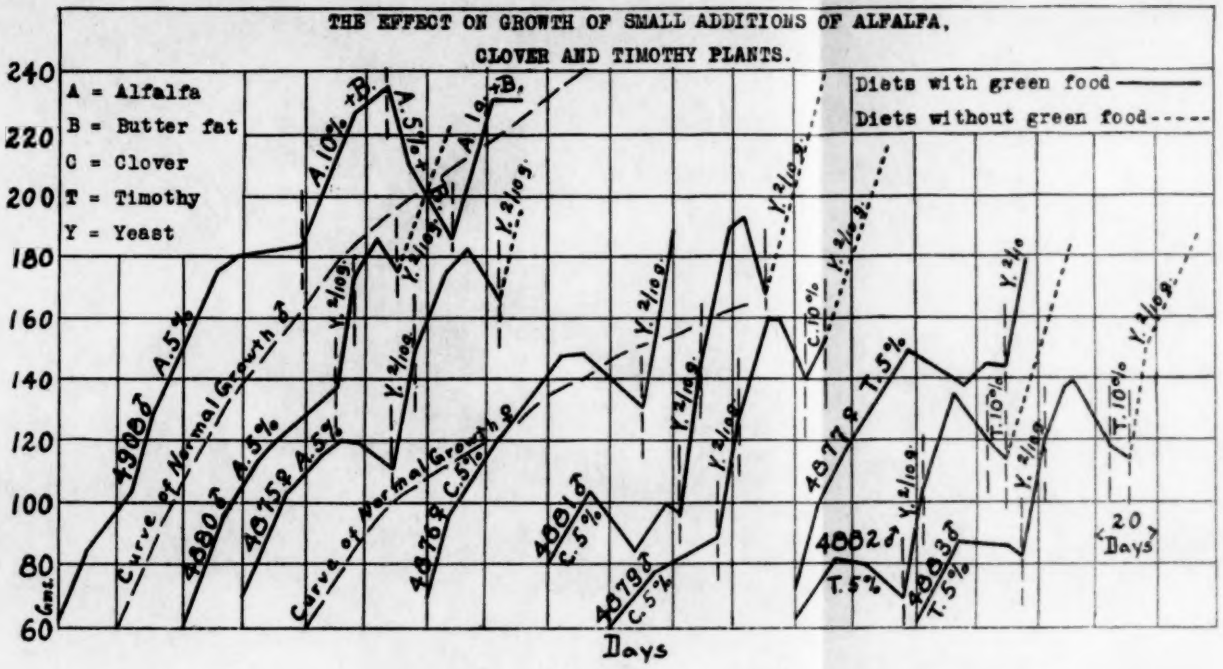
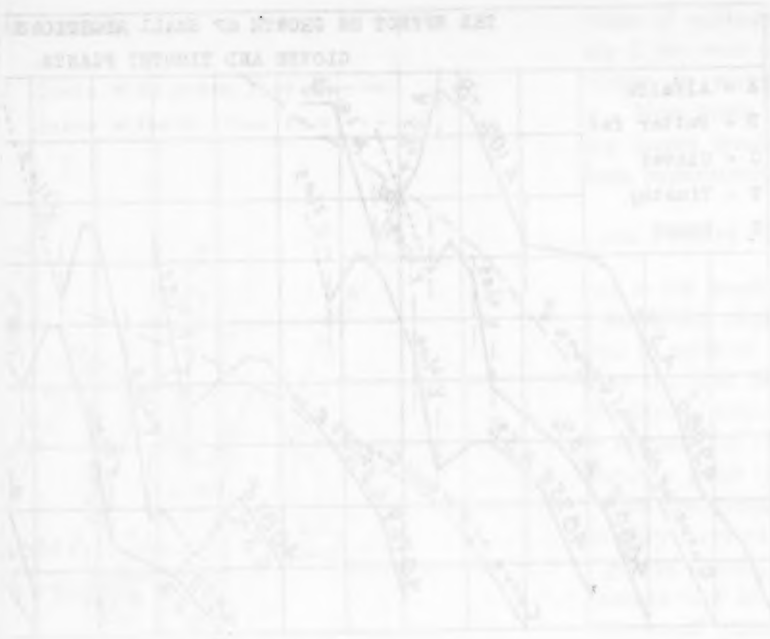


CHART III.

	Rats 4908, 4880, 4875			Rats 4876, 4881, 4879			Rats 4877, 4882, 4883		
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Alfalfa	10.0	5.0	0.0						
Clover				10.0	5.0	0.0			
Timothy							10.0	5.0	0.0
Meat residue	19.6	19.0	19.6	19.0	19.0	19.6	19.0	19.0	19.6
Salt mixture*	2.0	2.0	4.0	2.0	2.0	4.0	2.0	2.0	4.0
Starch	43.4	49.0	52.4	44.0	49.0	52.4	44.0	49.0	52.4
Lard	16.0	25.0 or 16.0	24.0 or 15.0	25.0	25.0	24.0	25.0	25.0	24.0
Butter fat	9.0	0.0 or 9.0	0.0 or 9.0						

* The preparation of this mixture is described in *J. Biol. Chem.*, 1913, xv, 317.



INFLUENCE OF HIGH TEMPERATURES AND DILUTE ALKALIES ON THE ANTINEURITIC PROPERTIES OF FOODS.

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From the standpoint of nutrition a knowledge of the stability of the water-soluble food accessory under the various conditions to which it may be subjected during the processes of food preservation and preparation is of importance. Individuals living on a mixed diet which includes fairly liberal amounts of raw fruits and vegetables without doubt are getting a considerable excess of the antineuritic vitamine; but the changing economic conditions have brought about an increased consumption of canned and dried foods. To what extent may the margin of safety be lessened by these dietary changes?

Reports of investigations dealing with the effect of high temperatures (100°C. or over) on this essential food constituent are contradictory. In certain instances the protective action against polyneuritis has been found to be destroyed in foods subjected to a temperature of 120°C. for a given length of time, while in others it apparently remains unaffected. For example, Grijns[1] found that small quantities of "Katjang-Idjo" beans (*Phaseolus radiatus*) boiled for 2 hours at 120°C. and added to peeled rice lost their power against polyneuritis in chickens. Similarly, meat, and to some extent unpeeled rice heated to 120°C. failed in their protective action. Eijkman,[2] however, reported that horse flesh boiled for 2 hours at this temperature did not produce polyneuritis in pigeons, although unpeeled rye, oats, millet, and barley, which had been boiled for 2 hours at 115°, 125°, and 135°C. respectively had little protective action. The antineuritic properties of wheat embryo and whey McCollum and Davis[3]

[1] Grijns, cited by Holst, A., *J. Hyg.*, 1907, vii, 619.

[2] Eijkman, C., *Virchows Arch. path. Anat.*, 1897, cxlviii, 523.

[3] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 247.

found were unimpaired after heating in an autoclave for 1 hour at 15 pounds pressure. But according to Chick and Hume[4] wheat embryo and yeast extract lose their potency at temperatures above 100°C. Exposure for 40 minutes to a mean temperature of about 113°C. decreases the efficiency about one-half as compared with the unheated control; exposure for 2 hours to 118–124°C. reduces their power to less than one-fourth. McCollum, Simmonds, and Pitz[5] were able to get normal growth on a ration in which navy beans, heated for 1¼ hours at 15 pounds pressure (120°C.), were the sole source of the antineuritic vitamine. Comparable results were obtained by Daniels and Nichols[6] with soy beans heated for 30 to 40 minutes at 15 pounds pressure. At 100°C. most observers have detected no destruction of the vitamine in neutral or acid media, although in dilute alkaline solutions it appears markedly affected at this temperature.

The explanation of the conflicting results in the vitamine-containing substances heated to the higher temperatures is not clear. It is possible that in certain instances small amounts of the antineuritic material were used. When the minimum amount is present a slight destruction would produce conspicuous results. It is also conceivable that the antineuritic vitamine may be present in tissues in two forms, one so bound chemically that it is unaffected by treatment which is destructive to the free or unbound form. Vedder and Williams,[7] and Williams and Seidell[8] have observed that acid hydrolysis of the alcoholic extract of rice polishings produced a substance more promptly curative in small doses than the original extract. The greater potency of the extract after hydrolysis may be due to the liberation of a portion which had been chemically bound. Recent studies by Cohen and Mendel[9] relative to the etiology of scurvy suggest that in certain instances an antiscorbutic vitamine may be similarly bound. Sprouted oats and barley were found to be more effective in preventing the onset of scurvy in guinea pigs than the unsprouted seeds. It is, moreover, not improbable that the

[4] Chick, H., and Hume, E. M., *Proc. Roy. Soc., Series B*, 1917–18, xc, 60.

[5] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1917, xxix, 521.

[6] Daniels, A. L., and Nichols, N. B., *J. Biol. Chem.*, 1917, xxxii, 91.

[7] Vedder, E. B., and Williams, R. R., *Philippine J. Sc., B*, 1913, viii, 175.

[8] Williams, R. R., and Seidell, A., *J. Biol. Chem.*, 1916, xxvi, 431.

[9] Cohen, B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxv, 425.

physiological structure of food may be a factor in determining the amount of destruction of the antineuritic vitamine, the starch and hemicelluloses of certain vegetables acting as protective material.

Studies dealing with the extracted antineuritic vitamine are of inestimable value in throwing light on the chemical nature of the complex, but from these one should not conclude that comparable changes take place in foods similarly treated. In order to know whether the observed phenomena are applicable, different types of foods which have been treated as far as possible in accordance with the household and commercial processes must be studied.

Since the usual household method of procedure consists in cooking vegetables in boiling water, while a temperature of 120°C. is most frequently used in the commercial canners and in the household pressure cookers, we have endeavored to determine whether the time required to cook certain types of vegetables under these conditions is sufficiently long to affect their antineuritic properties. Studies have also been made of the effect of cooking vegetables in dilute alkaline solutions at the boiling temperature, for the practice of adding small amounts of sodium bicarbonate (cooking soda) to the water in which such vegetables as beans, peas, and cabbage are cooked is not uncommon, in order to dissolve the intercellular material and decrease the time of cooking, or to preserve the natural color. This point is of particular interest, since the water in which foods are cooked in some sections of the country is permanently hard.

The vegetables used in the investigation were (1) cabbage, representative of the starch-free, succulent vegetable, containing small amounts of intercellular material which might, by adsorption, act as protective material to the vitamine; (2) beans, both soy and navy, foods containing considerable hemicelluloses and starch, which are most frequently cooked in the pressure cooker, or to which sodium bicarbonate is added.

In the investigation emphasis was laid on the difference in the antineuritic properties of beans cooked at high temperatures (approximately 120°C.) and those cooked in water to which dilute alkalies had been added. No comparisons were made between the cooked and raw beans since uncooked beans are not

a possible food for man. With the cabbage, studies have been made of the effect of raw cabbage and cabbage cooked under three different conditions; namely, (1) in water at 100°C., (2) in water at 120°C. for 15 minutes, and (3) in boiling water to which a slight excess of alkali had been added. The same amount of cabbage was used in all rations, the water in which the cabbage was cooked being included.

Comparable studies were made with purified rations to which the water-soluble vitamine from beans cooked at 120°C. for 20 and 40 minutes respectively were added, for it seemed probable, from the reports of other investigations, that the extracted food accessory is more sensitive to untoward influences than the unextracted material. Furthermore, it is of interest, particularly in relation to infant feeding, to know whether the liquor from vegetables similarly cooked has any antineuritic value. The possibility of using the vegetable liquor as a milk diluent suggests itself, for it has been shown by Osborne and Mendel[10] that the water-soluble food accessory in milk is not so high as we have hitherto believed. The dilution of cow's milk with water or a cereal diluent low in this food accessory may result in a food containing too little of the antineuritic vitamine for physiologic well being.

Young animals show the effects of inadequate diets much more quickly than adults. This is especially true of diets lacking the water-soluble food accessory. Therefore, in our study animals (rats) from 4 to 6 weeks old were selected for the trial feedings. All the diets, as far as possible, were comparable, containing equivalent amounts of physiologically good proteins, fat, inorganic salts, and carbohydrates. Since quantitative determinations of the fat-soluble vitamine have not been possible, the diets in all cases included an excess, as shown by the results of previous investigations. In addition to the amount supplied by the vegetables used, 5 gm. of butter-fat were added to every 100 gm. of dry ration. When the ration consisted of purified materials 8 gm. were used.

There was undoubtedly some variation in the amount of water-soluble food accessory present in the several diets; but in all

[10] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 537.

cases an excess, we believe, was included. For example, 100 gm. of the navy bean ration contained 50 gm. of navy beans. Similarly, 50 gm. of beans were used as the source of the water-soluble food accessory in the purified ration, and, although the method of extraction, in all probability, did not remove completely the antineuritic vitamine, it would seem as if there were an abundant supply present, since McCollum and coworkers[5] have produced normal growth in rats fed rations in which the water-soluble vitamine was obtained from 25 gm. of navy beans. In our soy bean rations 55 gm. were used.

While no data are available regarding the amount of cabbage necessary to furnish sufficient water-soluble vitamine for rats, McCollum and Kennedy[11] conclude from their work with polyneuritic pigeons that the material is present in rather low concentration. In our rations, in order to make sure that enough was included, 250 gm. of cabbage were used to every 100 gm. of dry ration.

The extracted water-soluble material from the beans was obtained in the following manner. The beans were soaked 12 hours, then autoclaved at 15 pounds pressure for 20 minutes in the case of navy beans and 40 minutes for the soy beans. This cooked material was then comminuted by pressing through a strainer, the liquor was filtered off, and the beans were washed several times with a liberal amount of distilled water. The filtrate and washings were then evaporated to small bulk, alcohol was added to precipitate the proteins, starch, and pectins; and the material was again filtered and the alcohol distilled off.

It is obvious that by this method no conclusions can be drawn with regard to the amount of destruction that may have taken place in the food accessory at the temperature employed. We were, however, not concerned with the question of how much destruction resulted, but whether the liquor from the foods, particularly beans, thus cooked, contains appreciable quantities of the antineuritic vitamine.

Recent studies of the effect of dilute alkalies at high temperatures on the extracted antineuritic vitamine have indicated that considerable destruction takes place. McCollum and coworkers[12]

[11] McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 491.

[12] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 55.

brought young rats to a standstill by feeding a purified ration lacking only the water-soluble vitamine. When the extract from 10 per cent of wheat embryo, which had been previously treated with a 0.28 per cent solution of sodium hydroxide at 100°C. for an hour, was added to the ration, the rats failed to grow. These authors concluded that the alkali had destroyed almost completely the antineuritic substance. In view of these results it seemed of interest to determine whether the extracted vitamine from the cooked beans was equally sensitive. Therefore in our experiments the water extracts of the beans prepared as described above were first made slightly alkaline to phenolphthalein with 0.1 N sodium hydroxide; an excess of 6.44 cc. of the sodium hydroxide solution was then added and the whole boiled in a reflux condenser for 30 and 60 minutes respectively. In all cases the water was distinctly alkaline to litmus before and after boiling.

In testing the effect of cooking vegetables with dilute alkali 33 cc. of a 5 per cent sodium bicarbonate solution were added for every 50 gm. of navy or soy beans, and 63 cc. for every 250 gm. of cabbage. The vegetables were cooked in distilled water until tender,—navy beans 90 minutes, soy bean 120 minutes, and cabbage 45 minutes. The excess alkali was neutralized with 0.1 N hydrochloric acid, and the vegetables were then combined with the other ingredients of the rations, the casein and butter fat being prepared in accordance with the usual methods.[5]

Since none of the vegetables in the amounts used supplied sufficient inorganic material for the experimental animals, one of the various salt mixtures was used in each ration to make the total inorganic content comparable to that of whole milk. A few sprinklings of ferric citrate were added to the purified rations, and two drops of a 2 per cent potassium iodide soution were added to the distilled drinking water once a week. The data concerning the composition of the various rations are given in Tables I and II.

The growth curves of the animals fed navy beans cooked at 120°C. for 20 minutes (Chart I, Group 1) as well as those fed beans cooked with an excess of sodium bicarbonate (Chart I, Group 2) indicate that in neither case was there any great destruction of the antineuritic vitamine. Growth in both groups

TABLE I.

Materials Used in 100 Gm. of Dry Ration.

Ration No.	Water-soluble food accessory.			Butter fat.	Casein.	Salt No.	Mixture.	Lard.	Cornstarch.	Filter paper.	Calories per gm.
	Source.	Amount of vegetable.	Preparation.								
		gm.		gm.	gm.		gm.	gm.	gm.	gm.	
I	Navy beans.	50	Autoclaved 20 min. at 120°C.	5	8	2	5.18	13	18.82		4.52
II	" "	50	Boiled with 33 cc. 5% $NaHCO_3$.	5	8	2	5.18	13	18.82		4.52
III	Soy "	55	Autoclaved 40 min. at 120°C.	5		3	3.46	10	26.55		4.61
IV	" "	55	Boiled with 33 cc. 5% $NaHCO_3$.	5		3	3.46	10	26.55		4.61
V	Cabbage.	250	Raw.	5	15	4	6.53	10	63.47		4.73
VI	"	250	Boiled at 100°C.	5	15	4	6.53	10	63.47		4.73
VII	"	250	Autoclaved 15 min. at 120°C.	5	15	4	6.53	10	63.47		4.73
VIII	"	250	Boiled with 62.3 cc. 5% $NaHCO_3$.	5	15	4	6.53	10	63.47		4.73
IX	Soy beans.	55	Extracted from beans autoclaved 40 min. at 120°C.	8	18	1	8.45	10	55.55	2	4.77
X	" "	55	Same as No. IX, but extraction boiled 30 min. with 6.4 cc. excess 0.1 N NaOH.	8	18	1	8.45	10	55.55	2	4.77
XI	Navy beans.	50	Extracted from beans autoclaved 20 min. at 120°C.	8	18	1	8.45	10	55.55	2	4.77
XII	" "	50	Same as No. XI, but extraction boiled 30 min. with 6.4 cc. excess 0.1 N NaOH.	8	18	1	8.45	10	55.55	2	4.77
XIII	" "	50	Same as No. XI, but extraction boiled 60 min. with 6.4 cc. excess 0.1 N NaOH.	8	18	1	8.45	10	55.55	2	4.77

TABLE II.

Composition of Salt Mixtures.

Salt mixture No.	NaCl	KCl	$CaHPO_4$ $2H_2O$	$CaCl_2$	K citrate.	$MgSO_4$ $7H_2O$	Mg citrate.	Ca lactate.	Total.
1	0.876	0.3012	3.611	0.1876	2.6157	0.4255	0.4331		8.446
2	0.763		2.4191	0.4783	2.2139			0.2435	5.298
3	0.342		1.6277	0.8925	0.0201			0.5763	3.459
4	0.859	0.3868	3.355	0.0683	1.3692		0.496		6.534

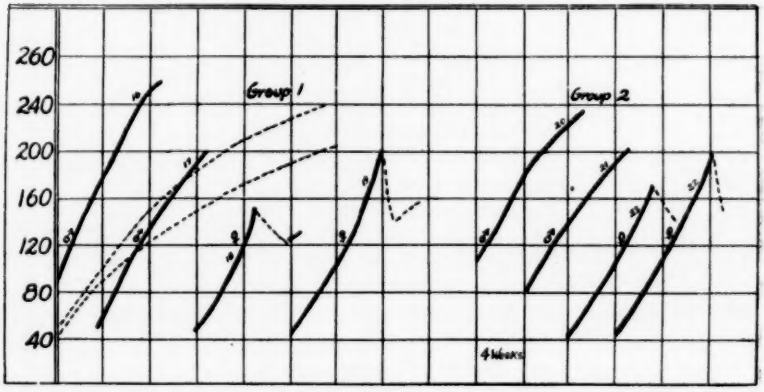


CHART I. The growth curves of animals (Group 1) fed rations in which the only source of the antineuritic vitamine was navy beans heated for 40 minutes at 120°C. (Ration I) give no indication of vitamine destruction. Nor do the curves of growth of animals (Group 2) receiving rations containing navy beans boiled in a dilute alkaline solution (Ration II) show a decrease in the potency of the vitamine.

was rather remarkable as compared with that of rats usually considered normal. Our animals on the bean rations not only grew more rapidly but reproduced at an early age.

The curves of growth of animals (Chart II) fed the rations containing the soy bean which had been treated the same way as the navy beans also indicate that neither high temperatures (Group I) nor dilute alkalies at the boiling temperature (Group 2) are very detrimental to the antineuritic vitamine in foods. As in the case of the navy beans, the growth curves surpass those usually considered normal. Similarly, reproduction occurred at an early age and the young were successfully reared.

Apparently the antineuritic vitamine of beans cooked in a pressure cooker, commercially canned beans, and beans made soft by the addition of sodium bicarbonate is not greatly affected by these processes.

The results of the feeding experiment with cabbage (Chart III) are of particular interest, since comparisons have been made between animals fed raw cabbage (Group 1) and those fed cabbage cooked in the pressure cooker (120°C.) for 15 minutes (Group 2); boiled in distilled water 45 minutes (Group 3); and

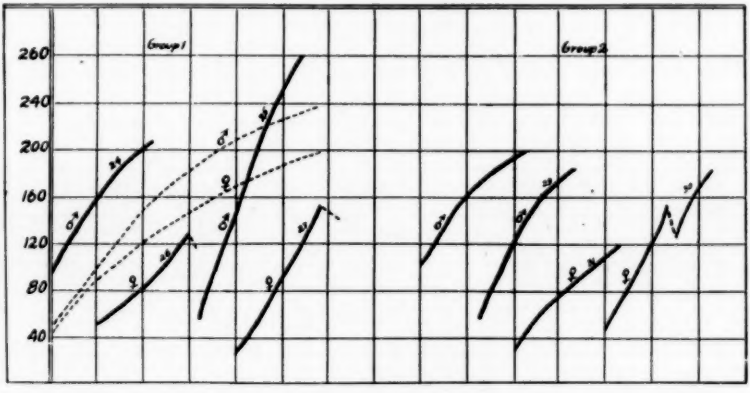


CHART II. The antineuritic vitamine of soy beans apparently is not greatly affected by cooking the beans in an autoclave at 15 pounds pressure for 40 minutes, nor by cooking at the boiling temperature in a dilute alkaline solution. The animals (Group 2) receiving the soy beans boiled with sodium bicarbonate (Ration IV) grew as well as those (Group 1) fed beans cooked at the higher temperature (Ration III).

boiled in a dilute alkali 30 minutes (Group 4). The growth curves of all are similar and give no indication that there was considerable destruction of the vitamine, resulting from the various methods of treatment. It is possible that longer cooking under similar conditions would have produced different results, but since our aim was to determine the effect on the antineuritic vitamine in vegetables prepared in accordance with the usual household and canning processes, there seemed no point in longer treatment.

Our failure to obtain results indicating that high temperatures and dilute alkalies at the boiling temperature are destructive to the antineuritic vitamine apparently was not due to the fact that a large part of the vitamine was held in the vegetable and therefore was unaffected by the method employed. The animals on the purified rations which contained the extracted water-soluble food accessory from the autoclaved soy and navy beans also made normal growth. Even the animals which received the

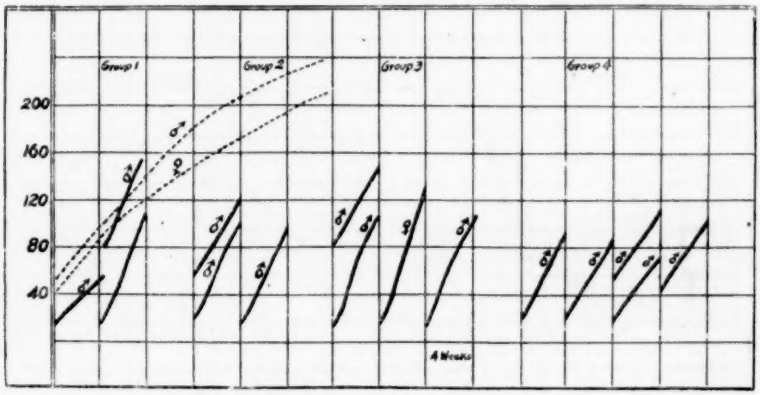


CHART III. The antineuritic vitamine of cabbage is apparently unaffected by the usual cookery processes. The animals of Group 1 received a ration in which raw cabbage (Ration V) supplied the antineuritic vitamine. Group 2 was given a similar ration which included cabbage cooked in boiling water (Ration VI). The cabbage in the ration of Group 3 was cooked at 120°C. for 15 minutes (Ration VII), while in Group 4 sodium bicarbonate was added to the water in which the cabbage was cooked (Ration VIII).

extra vitamine boiled with dilute alkali for 30 and 60 minutes respectively gave little evidence of vitamine destruction. During Period 1 (Charts IV and V, Groups 1 and 2) the failure of our animals to grow on the purified rations was undoubtedly due to an insufficient amount of the antineuritic vitamine. The rations at this time included the cold alcoholic extract from 9 per cent of raw beans. The addition of the water-soluble vitamine from a larger proportion of cooked beans stimulated growth (Periods 2). The curves during Periods 1 have been included to meet the

possible criticism that the casein supplied a sufficient amount of the vitamine to meet the needs of the animals.

Our results with the water-soluble food accessory treated with dilute alkali are not in accord with those reported by McCollum, and Simmonds.[12] Their animals receiving the water-soluble vitamine boiled for 60 minutes in an alkaline medium made prac-

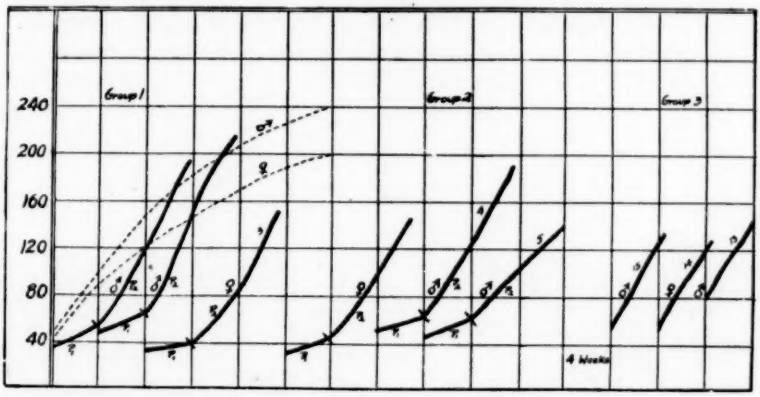


CHART IV. During Period 1, Group 1 received rations in which the water-soluble food accessory was obtained from the alcoholic extract of 9 gm. of raw navy beans, and Group 2 received a similar ration in which the food accessory had been boiled for 30 minutes with dilute alkali. An increase in the antineuritic vitamine stimulated growth in all cases. During the Periods 2, Group 1 received Ration XI containing the water-soluble vitamine from 50 gm. of navy beans, autoclaved at 15 pounds pressure for 40 minutes; Group 2 was given Ration XII, containing the same amount of the water-soluble food accessory boiled 30 minutes with a dilute alkali; and Group 2 received a similar ration (Ration XIII) containing the water-soluble material boiled 60 minutes with dilute alkali. The curves of growth suggest that the vitamine has not been materially affected by the treatment employed.

tically no growth. It is probable, however, that their rations contained the minimum amount of the antineuritic vitamine, having been obtained from 10 per cent of wheat embryo, whereas in our foods a considerable excess of the vitamine was used, and the slight destruction evidenced by the lack of growth in their animals has not been made apparent in our investigation.

The fact that our animals made normal growth on rations in which the liquor from the cooked beans was the sole source of

the water-soluble vitamine emphasizes again the undesirability of discarding the water surrounding cooked or canned vegetables. This contains not only much of the inorganic constituents of the vegetables, the soluble carbohydrates, and proteins, but, obviously, much of the water-soluble food accessory as well.

The note of warning sounded by Chick and Hume[4] to the effect that grave danger may attend the use of large amounts of tinned foods, we believe, is unfounded, at least from the standpoint of the antineuritic vitamine content of the food. Bitting[13]

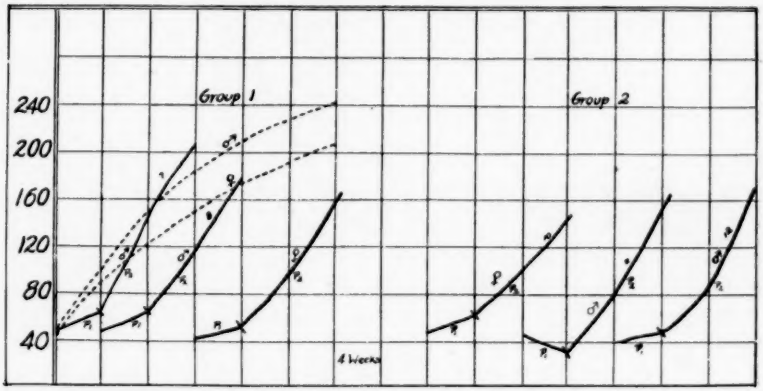


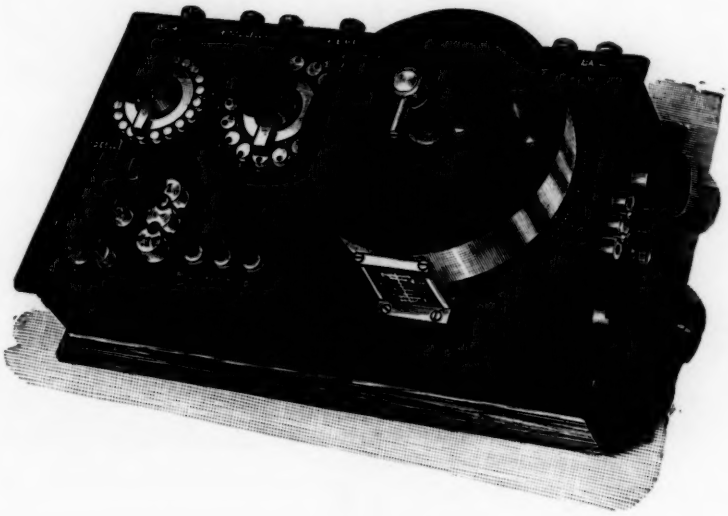
CHART V. During Period 1 the animals (Groups 1 and 2) were given rations consisting of purified foods in which the antineuritic vitamine was obtained from ground raw soy beans. The change (Period 2) to the water-soluble vitamine from 55 gm. of soy beans autoclaved at 15 pounds pressure for 40 minutes (Ration IX) produced normal growth in Group 1. Similarly, the addition of a larger amount of the water-soluble vitamine (Period 2) which had been boiled with an excess of alkali for 30 minutes (Ration X) stimulated growth in Group 2.

reports that fruits are usually processed at the boiling temperature from 12 to 15 minutes; corn at 118–120°C. for 75 to 80 minutes; green beans, lima beans, peas, and spinach at 115–118°C. for 30 to 40 minutes; and pumpkin at 120°C. for 90 minutes. From our results as well as those of McCollum and coworkers, it seems improbable that in the commercial canning of foods this vitamine is destroyed to such an extent that too little will

[13] Bitting, A. W., *U. S. Dept. Agric., Bull. 196*, 1915.

be included in the diet where the usual amount of canned food is eaten. Even if there should be a considerable increase in the amount of canned food consumed, we believe that there will still be present enough of the antineuritic vitamine to meet the physiologic requirements, provided the diet is not materially changed in other respects. Decreasing the time required for softening beans by the use of pressure cookers or the addition of small amounts of dilute alkalies is justified, since the antineuritic vitamine of vegetables does not appear to be greatly affected by these processes.

A POTENTIOMETER FOR THE DETERMINATION OF HYDROGEN ION CONCENTRATION IN SOLUTIONS



THE POTENTIOMETER illustrated above is now being used by a number of investigators for measuring the E. M. F. between the Hydrogen and Calomel electrodes in Hydrogen Ion Concentration determinations. It is rapidly replacing the electrometer for such work for the following reasons: first, it is much more sensitive than the electrometer; second, its use does not entail the annoying delays met with in the use of the electrometer when overcharged and thus polarized.

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